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# PLANT CHEMOTHERAPY As Evaluated By The Fusarium Wilt Assay on Tomatoes

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THE CONNECTICUT AGRICULTURAL EXPERIMENT STATION
NEW HAVEN

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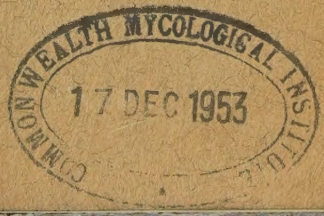

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*by*

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# Plant Chemotherapy as Evaluated by the Fusarium Wilt Assay on Tomatoes

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## I. Introduction

Plant chemotherapy offers the possibility of controlling types of plant diseases that are difficult to combat with established techniques. For this reason, the chemotherapeutic approach has intrigued the research plant pathologist for many years. For some time research on plant chemotherapy has been actively investigated in this laboratory and at the Rhode Island Agricultural Experiment Station, and a number of publications have reported the results of these studies. As other laboratories have contributed to this rapidly widening field, plant chemotherapy has become a broader subject, involving several apparently unrelated approaches.

The present bulletin attempts to evaluate the several approaches to chemotherapy as an integrated whole. In order to do this, it is necessary first to present a considerable body of experimental evidence obtained from the Fusarium wilt assay. For this reason the assay method is described in detail, together with the experimental basis for its design. Then follows a survey of the kinds of information which have been obtained about chemotherapeutants from its use. This information, together with what is available from other studies on the subject, is used to evaluate the three principal lines along which research is developing. In this way, the subject appears unified by common objectives and by mutually unsolved problems for future research to explore.

### A. DEFINITIONS

In accordance with usage, *disease* is defined as a harmful deviation from normal physiological processes, arising from a continuing irritation or deficiency in the plant (1). A plant is *inoculated* when the pathogen first contacts its host and becomes *infected* when the pathogen has established itself and grows upon or within the host, using it as a substrate. A *disease* begins when an infection is established, whether or not injury to the host is yet visible or measurable.

Chemotherapy by definition involves the use of compounds. Accordingly, *plant chemotherapy* is the control of plant disease by compounds that, through their effect upon the host or pathogen, reduce or nullify the effect of the pathogen after it has entered the plant (28).

The compound that initiates this effect, either directly or indirectly, is called a *chemotherapeutant.* Undoubtedly some compounds will be altered in the soil or after entering the plant. The altered product, rather

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than the original compound, may participate directly in chemotherapy. However, this product may not be useful as a chemotherapeutant if applied to the plant.[1]

Chemotherapeutants cause a reduction in disease severity in various ways. The term chemotherapeutant is a general one, describing any compound that is useful in alleviating disease by means of chemotherapy. Recognized types of chemotherapeutants are: (a) compounds that antidote toxins formed by the pathogen, but do not act primarily on the pathogen itself, (b) compounds that alter the metabolism of the host so that its resistance to infection is increased, and (c) systemic fungicides. The systemic fungicide has a known and restricted mode of action: it enters the plant, is translocated as far as the locus of infection (or the potential locus) and then acts directly upon the pathogen by virtue of its fungitoxic properties.

A chemotherapeutant may be used either to prevent disease or to cure it. It has been said that in reality two types of treatment should be distinguished: chemical prophylaxis which prevents disease, and chemotherapeutic treatment which cures it (52). Such a distinction was at one time proposed from this laboratory (30), but was later abandoned. Useful as it may be in theory to make this distinction, one is confused by it in practice. Penicillin is generally used to cure an infection and is called a chemotherapeutant. But when it is administered by a doctor to protect the patient exposed to pneumonia, must it be called by a different name and be considered to act differently? If infection begins and penicillin kills the pneumococcus, there is no essential difference in the biology of the situation than when penicillin is used in an advanced case of pneumonia, so far as mode of action is concerned. In the one case the inoculum potential is small; in the other it is large. The difference between prevention and cure is a matter of time relative to when inoculation occurs, and in experimental work it may be impossible to say whether or not infection has occurred. In practice the distinction tends to become a matter of the intent to cure or prevent disease when the plant was treated. If the operator cannot say surely whether the plant was infected at that time, he is in a dilemma! The practising physician has long since realized that such a distinction is a hindrance to clear thinking and has come to use therapy in a preventive sense when it is a matter of convenience. Such words from the medical field as immunotherapy (43) indicate the problem. Vaccines and the immune reaction are techniques of preventive medicine.

## B. BACKGROUND OF THE PRESENT STUDY[2]

The present study arose out of interest stimulated by three discoveries in chemotherapy. Each of these represented a boldly different

[1] The term fungicide is used in the same sense. What is applied to the plant may not be what kills the fungus spore. But the ultimately active ingredient may be worthless if used under field conditions in the conventional way. Thus, Rich and Horsfall have shown that a volatile product is formed from disodium ethylene bisdithiocarbamate that is more toxic to fungi than the original compound and they suggest that it is this volatile product that is effective against the fungus spore (44). This volatile product would be useless as a protectant fungicide if applied as a spray to plants.

[2] Since the literature on chemotherapy of plant diseases has been adequately reviewed (28, 29, 39, 46, 59), no attempt will be made to do so here.

approach to the subject and each indicated that it was possible to control diseases in practice by chemotherapeutic techniques.

The first of these was a study by Howard (31) on the bleeding canker disease of hard maples. The pathogen, *Phytophthora cactorum*, was shown to affect the host by producing a toxin that caused wilting of leaves. Howard conceived of the original approach of antidoting the toxin by compounds which might be injected into the tree. He obtained the toxin in culture filtrates and showed that it was neutralized when mixed with diaminoazobenzene dihydrochloride. Thus, cuttings placed in raw culture filtrates wilted severely, while cuttings placed in filtrates to which a small amount of the compound had been added failed to wilt. When solutions of the dye were injected into diseased trees, the latter recovered from wilting symptoms (31).

An equally significant piece of work was carried out by Zentmyer and Horsfall (67, 68) on the chemotherapy of Dutch elm disease. They tested a number of compounds for fungitoxicity to *Ceratostomella ulmi* and injected them into elms. Certain of these fungitoxic compounds caused diseased elms to recover from wilting. Outstanding among them were 8-quinolinol sulfate and the corresponding benzoate. Subsequent studies showed that significant reductions in the incidence of natural infections by *C. ulmi* could be demonstrated in plots of trees that were treated as compared with plots of trees that were not (11).

At about the same time Stoddard (57) published his work on the chemotherapy of X disease of peach. This disease is caused by a virus and can be transmitted by grafting diseased buds into healthy stock. Stoddard showed that virus in diseased buds can be inactivated by soaking them in solutions of various compounds (55). The next step in Stoddard's investigations was to bud diseased material into healthy trees, and inject solutions of such compounds into them (56). He thus showed chemotherapy of X disease was possible both for buds and for whole trees (57).

Horsfall (26) pointed out in 1945 that a useful chemotherapeutic mechanism involved the use of specific fungicides within the host and discussed a number of instances where fungicides acted systemically in the plant and acted specifically upon the pathogen to cure disease.

Thus, previous work had indicated that plant diseases might be controlled chemotherapeutically to a practical degree. When promising compounds were applied to the soil and absorbed by roots, chemotherapeutic effects on Dutch elm disease were both more uniform and of greater magnitude than when they were injected into the stem (11). The next problem, therefore, was to develop an assay which would quantitatively measure the chemotherapeutic potency of compounds. This was undertaken as the first objective of the present study.

## II. An Assay Method for Chemotherapeutic Potency

Fusarium wilt of tomatoes was chosen as the disease on which the assay method was based because of the suitability of tomatoes to greenhouse cultivation, and the ease with which *Fusarium oxysporum* f. *lycopersici* can be grown and inoculated into plants.

### A. METHOD OF GROWING PLANTS

Bonny Best variety of tomatoes was chosen, since it is highly susceptible to Fusarium wilt, provides a series of uniform plants, and is readily available. Two-week-old seedlings are transplanted into four by four-inch veneer wood boxes containing builders' sand. From this time until plants are discarded, they are maintained at a nutritional level optimal for symptom expression by Fusarium wilt (58). Double strength Hoagland's solution (25) plus micronutrients is applied three times weekly, the quantity being slightly in excess of what is retained by the container filled with sand.

To simplify frequent application of nutrient solution, a liquid proportioner is used in the form of a Hayes Jr. sprayer (Figure 1). This is modified by removing the nozzle (parts 1 and 2 of Figure 1), inserting a short copper tube of proper diameter in its place, and attaching rubber tubing over the copper tube to aid delivery of solution to the plant. The proportioner is operated from the city water line and is calibrated for both proportioning rate and delivery rate. The stock solution of nutrient is made correspondingly more concentrated to allow for the proportioning rate. Delivery of 50 ml. of diluted nutrient to each plant is controlled by timing the delivery at a known delivery rate.

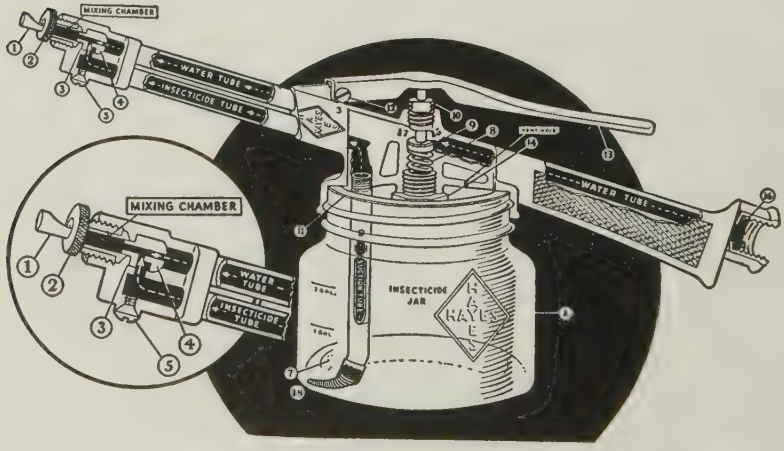


**Figure 1. The Hayes Jr. Sprayer. (Illustration furnished through the courtesy of the Hayes Spray Gun Company, Pasadena, California.)**

To insure the production of uniformly susceptible plants, they are grown in a greenhouse which is maintained at 80°F. Constant temperature is maintained by thermostatic control of the steam line and by automatic ventilation. In summer, when temperatures rise above 80°F., temperature is maintained as close to the ambient temperature as possible through the use of shade and adequate ventilation.

Greenhouse benches are watered automatically, in accordance with the Cornell system (42). Here, water levels in the greenhouse bench are maintained by means of a float valve. Control of this factor has very markedly reduced variability between plants.

## B. PRELIMINARY EXAMINATION OF COMPOUNDS

### 1. Formulation

No formulating agents are added to the compound under test as a chemotherapeutant. This convention was adopted for two reasons. Formulating agents are frequently more phytotoxic than the compound under test in such experiments, and only serve to confuse the estimation of phytotoxic levels of the compound itself. Moreover, formulating agents may themselves have chemotherapeutic activity, thus causing an erroneous estimate of the activity of the compound under test. As an example, isopropyl alcohol, which is frequently used as a cosolvent in handling aqueous solutions of organic compounds, reduces the severity of Fusarium wilt in tomatoes.

### 2. Measuring Phytotoxicity of Compounds

The first operation carried out with a new compound is to measure its phytotoxicity to the test plant. A dilution series of the compound is prepared and the roots of tomato seedlings are immersed in each solution. After 48 hours, the plants are read for phytotoxic symptoms and the maximum nonphytotoxic concentration is noted. This is the concentration which is used on plants in the first test of chemotherapeutic activity. If severe phytotoxicity appears in plants in the standard assay, subsequent tests are made with less concentrated solutions.

## C. AN ASSAY OF CHEMOTHERAPEUTIC POTENCY

### 1. Method of Applying Compounds to Plants

Usually, several compounds are assayed simultaneously in an experiment. Plants are arranged in either a Youden square (66) or randomized block during the treatment period and also during the incubation period.

Because treatments are randomized, it is essential to eliminate any cross-contamination of compounds during the treatment period. Contamination is prevented by reserving one empty greenhouse bench for chemotherapeutic treatment. Across its top are laid iron rods which support a wire screen of one-inch mesh. Plants undergoing chemical

treatment are set upon this screen and excess solution drains into the bench. If clay pots are used for growing plants, they may become contaminated with compounds used in previous experiments. For example, in one experiment clay pots were used during treatment with a compound that produces a formative effect. Even though washed before reuse, these pots contained enough of the compound to produce formative effects on plants subsequently grown in them. For this reason expendable veneer boxes are used in which to grow plants. The sand and veneer boxes are discarded after use in one experiment.

Stock solutions of the test compounds are prepared in adequate volume to serve for the entire experiment, at strengths which were previously determined as being the maximum concentration tolerated by the plant without serious phytotoxicity. When compounds are insoluble at the desired concentration, suspensions are used. A Hayes Jr. spray gun has been modified to speed the application of compounds to plants. The central piston and springs in the hand valve (parts 8, 9, 10 and 14 in Figure 1) are removed and a rubber stopper is placed under the handle. The output water tube is then plugged. The screw in the "insecticide tube" (part 5, Figure 1) is then removed and a short copper tee is soldered in this position. The stem of this tee is of small diameter and curved upward, and serves to stop the flow of solution immediately when the valve is released. To the open end of this tee is attached a length of rubber tubing, which is used to guide the flow of solution around the base of the plant under treatment. The gun is then connected to a compressed air line, and air displaces the solution directly from the container of the gun. When air pressure is constant, the delivery rate of solution is also constant, and the volume delivered can be controlled by timing the operation.

When a compound is to be applied, a quantity of the test solution slightly in excess of what is required is placed in the container of the gun. The pressure in the air line is carefully adjusted and 50 ml. of solution is delivered to each plant designated to receive that treatment. The gun and container are then rinsed out and the next test solution is placed in the container. This procedure is both rapid and accurate in the hands of a skilled operator.

Vigorous six-week-old plants receive one such treatment each day for ten days. Apart from this treatment, plants are watered only as required to prevent wilting or as they receive their regular applications of nutrient solution three times a week. The 50 ml. of solution applied each day is slightly more than the plant and its container will retain. Thus, the solution bathing the roots is replenished daily. Check plants, of course, receive a like amount of water.

### 2. Inoculation of Plants with Fusarium

Inoculum is prepared by growing shake cultures of *Fusarium oxysporum* f. *lycopersici*[1] in a casein hydrolysate medium (11) for four days. At the end of this time the culture will consist predominantly of bud cells

[1] The strain of *Fusarium oxysporum* f. *lycopersici* used in these studies was obtained from Dr. J. C. Walker and is the virulent strain used in their research on the relation of plant nutrition to disease development (64).

and will contain approximately $10^8$ cells/ml. This culture is diluted with an equal volume of distilled water before using it for inoculum on plants.

The day after treatment of plants with compounds is completed, plants are inoculated with *Fusarium*. After plants are uprooted, the roots are washed thoroughly, and about one-third of the root system is forcibly torn away. The injured root system of the plant is immersed for a few seconds in the dense suspension of *Fusarium* cells, after which the plant is potted in fresh sand in a container that has never been in contact with chemotherapeutant.

The steps in the inoculation procedure are related to the objectives of the chemotherapy assay. Washing of roots before inoculation is intended, not only to remove as much sand as possible, but also to remove any compound clinging to the surface of the root system. This operation tends to assure that any protection the plant has against infection by *Fusarium* arises from the effects the compound has produced inside the plant and not from the compound upon the root surface only. Injuring of roots not only makes infection by *Fusarium* certain, but exposes plant surfaces that have not been in external contact with chemotherapeutant previously. If infection fails to occur through these surfaces, a chemotherapeutic effect has occurred.

The inoculated plants are now placed in an automatically watered greenhouse bench that has had no contact with chemotherapeutants. From this time, nutrient is applied to the plant three times weekly. Plants are held until disease symptoms develop in the check plants.

### 3. Grading the Severity of Disease

When symptoms of disease are well developed in the inoculated check plants, the severity of disease in plots is assessed in terms of vascular discoloration. In the stem there are usually six primary vascular bundles; three large ones which alternate with three small ones. As we shall see, it is unnecessary to grade intensity of discoloration and one actually loses information by attempting to determine whether the three small vascular bundles are discolored or not.

At each internode, starting with the bottom of the stem, the number of large discolored vascular bundles is recorded. These observations are summed and divided by the total number of bundles observed (three times the number of internodes). The resulting index ranges from 0 for the healthy plant to 1 for the plant that is dying of Fusarium wilt.

Plants are graded in order along the columns on the greenhouse bench so that grading of treatments is at random.

### 4. Analysis of Data

The vascular index is computed for each plot and these are analyzed for variance. Where applicable, Yate's method of analysis for incomplete Latin squares is used (4); otherwise the analysis is the familiar one for randomized blocks.

## 5. Summary of a Typical Experiment

By presenting results of a typical experiment in which the activity of a series of compounds was explored, it is possible to indicate the extent to which this assay yields quantitative results. In the experiment under discussion, 21 treatments were applied, each to five replicate plants. The treatments consisted of 19 compounds plus an inoculated and an uninoculated check.

Each compound was applied at a single concentration, the maximum strength at which it was judged by preliminary trial to be nonphytotoxic. For the sake of simplicity, the treatments are designated by letters only. The inoculated check was treatment U and the uninoculated check was treatment T.

After disease symptoms were well developed in the inoculated check, plants were graded for disease severity. The appearance of a representative plant receiving treatment with an effective compound and of a check plant is shown in Figure 2. In analyzing results, the missing plot technique had to be used to assign grades to two plots for treatment E, which was still highly phytotoxic at the concentration employed. Yate's method of analysis was found inapplicable, and so the variance was analyzed by the usual analysis for randomized blocks, with the results shown in Table 1.

**Figure 2. The effect of chemotherapeutic treatment in reducing the severity of Fusarium wilt of tomato. The plant on the right was treated with the potassium salt of 2-carboxymethyl mercaptobenzothiazole, HD 109, (Treatment P) before inoculation and the plant on the left was similarly treated with water (Treatment U).**

TABLE 1. THE VARIANCE OF VASCULAR GRADES IN A CHEMOTHERAPY ASSAY

| Source of variance | Degrees of freedom | Mean squa.e | Variance ratio |
|---|---|---|---|
| Total | 104 | | |
| Blocks | 4 | 0.1090 | 3.46* |
| Treatments | 20 | 0.6005 | 19.06** |
| Error | 78[1] | 0.0315 | |

* Difference significant with odds of 1 in 20.
** Difference significant with odds of 1 in 100.
[1] Two degrees of freedom lost because of two missing plots in treatment E.

Quite apparently there was a highly significant effect of treatment by compounds on the severity of Fusarium wilt and also an effect of position of plants across the width of the greenhouse bench.[1] The performance of treatments is shown in Table 2. Plants under treatment by twelve[2] of the compounds were less severely diseased than check plants by an amount greater than the least significant mean difference.

TABLE 2. EFFECT OF CHEMOTHERAPEUTIC TREATMENTS ON SEVERITY OF FUSARIUM WILT OF TOMATO

| Treatment | Mean grade |
|---|---|
| J | 0.00** |
| I | 0.06** |
| C | 0.07** |
| P | 0.10** |
| T | 0.10** |
| K | 0.11** |
| N | 0.22** |
| A | 0.23** |
| E | 0.28** |
| D | 0.45** |
| G | 0.58** |
| H | 0.63* |
| R | 0.74 |
| F | 0.79 |
| M | 0.79 |
| S | 0.79 |
| Q | 0.83 |
| O | 0.88 |
| U | 0.89 |
| B | 0.91 |
| L | 0.93 |
| Least Significant Mean Difference | |
| P = 0.05 | 0.22 |
| P = 0.01 | 0.30 |

* Treatment differs significantly from inoculated check (U).
** Treatment differs with high significance from inoculated check (U).

[1] Positional effects of plants in the greenhouse bench frequently are not significant.
[2] This experiment contained a much higher proportion of effective treatments than are found in a random sample of compounds. Effective and ineffective treatments were deliberately selected to cover the range of responses in an effort to determine the value of several possible grading methods. The latter topic is discussed in Section IV, pages 29-48.

Treatment H reduced disease significantly and resulted in a mean grade which was about 0.7 that of the inoculated check (U). Thus, the assay method discriminates differences much smaller than are of practical interest, and is sufficiently sensitive to be useful in assaying chemotherapeutants.

## III. Experimental Basis for the Assay Procedure

A straightforward procedure has been given for the chemotherapy assay as it is used currently. Actually this procedure has been modified in its details over the six years it has been used, whenever experiments indicated that a change would be advantageous. The experimental evidence behind steps in the procedure is presented below.

### A. TIMING OF CHEMOTHERAPEUTIC TREATMENT RELATIVE TO INOCULATION WITH FUSARIUM

Chemotherapeutic effects are greatest on the X disease of peach when compounds are applied to plants after inoculation (57). Similarly, when beans are inoculated with the bean blight organism, *Xanthomonas phaseoli*, more effective control was achieved by chemotherapeutic treatment after inoculation (12).

In the case of Fusarium wilt of tomato, chemotherapeutic effects are greatest when this order is reversed. The results of a single experiment will suffice to illustrate this point, although these results have been confirmed using several compounds. A solution containing 16 ppm of CC 1182 (4-chloro-3, 5-dimethyl phenoxy ethanol) was applied to plants as a chemotherapeutic treatment for the standard 10-day period. One lot of six plants was treated and inoculated on the day following the last application of compound. Another lot of plants was inoculated first, and treatment was begun the same day. In another lot, treatment was begun the day following inoculation. Similarly, treatment on lots of six plants each was begun two and four days after inoculation. After a suitable incubation period, the plants were graded for severity of Fusarium wilt in terms of vascular discoloration. The mean grades for each series of plants is shown in Table 3.

Table 3. The Effect of Time of Chemotherapeutic Treatment Relative to Time of Inoculation Upon the Effectiveness of Treatment

| Days between time of inoculation and first application of chemotherapeutant | Severity of disease[1] |
|---|---|
| −11 | 0.21 |
| 0 | 0.89 |
| 1 | 0.99 |
| 2 | 1.00 |
| 4 | 1.00 |

[1] This is the grading described on page 13. Other grading systems are discussed in Section IV, pp. 29-32.

The only time when treatment was really effective in reducing the severity of Fusarium wilt was when it was applied prior to inoculation with *Fusarium*. Applications made after inoculation were less effective. Furthermore, the longer the period after inoculation that treatment is begun, the less effective is the treatment. For this reason treatments are applied prior to inoculation in the standard assay.

## B. METHOD OF APPLYING COMPOUNDS TO PLANTS

When a chemotherapeutant is injected into the trunk of an elm for control of Dutch elm disease, its distribution is spotty, whereas when it is applied to the soil around feeding roots and then absorbed, distribution is more uniform (46). Further evidence for this is based upon the distribution of 8-quinolinol benzoate in elms, as detected through development in the plant of a color reaction between 8-quinolinol and ferric sulfate. Moreover, reduction in severity of disease is less erratic and effects are more pronounced when compounds are absorbed by the root system than when they are injected into the stem (11).

A more quantitative study of this relationship was made on the chemotherapy of bacterial blight of beans (12) and the results were confirmed. Consequently, in the present investigation no further attempt was made to illustrate this principle again and compounds were applied to the sand in which plants were grown.

## C. INFLUENCE OF AMOUNT OF COMPOUND ABSORBED UPON THE CHEMOTHERAPEUTIC RESPONSE OF PLANTS

The assay procedure calls for treating plants with the chemotherapeutant for 10 days before inoculation. This procedure is based upon studies of the chemotherapy of bacterial blight of beans in which it was shown that, as the number of applications of chemotherapeutant was increased up to eight, the chemotherapeutic effects became more pronounced (12). It seems reasonable, moreover, to assume that a certain minimum of chemotherapeutant must be absorbed by the plant before any effects will be noted. Similarly, beyond a certain maximum absorption, there should be little detectable difference in response of plants to additional treatment. To apply compounds beyond this stage is inefficient, but the procedure should involve an application schedule which approaches this maximum, as measured by the response of plants. If this is done, it will matter little whether the number of applications is ideal or slightly in excess of what is necessary. There is some experimental evidence to indicate that a 10-day application schedule may be more than is necessary. For example, an experiment was conducted in which two chemotherapeutants, HD 3 (*n*-octadecyl trimethyl ammonium pentachlorophenate) and CC 1182 (4-chloro-3,5-dimethyl phenoxy ethanol) were applied to tomatoes, each at a concentration of 8 ppm, for varying times. Plants of standard age for treatment were uprooted, the roots washed and immersed in solutions of these two compounds. After two, four, and six days, five plants were removed from each solution, inoculated in the usual manner and planted in sand. All plants were inoculated on the same day and from the same batch of inoculum. Disease severity was noted three weeks after inoculation. In this experi-

ment severity of Fusarium wilt was based upon grading leaves individually on the plant on a scale ranging from 0 (healthy leaf) to 4 (completely wilted), summing the grades for the individual leaves and dividing by the number of leaves observed. The resulting mean leaf grade[1] for treated plots is expressed in Table 4 as a percentage of the mean leaf grade for the corresponding check plots. These data, together with information on the volume of solution absorbed by the five plants in the treatment period, are presented in Table 4.

Table 4. The Effect of the Amount of Chemotherapeutant Absorbed by Plants Upon the Severity of Fusarium Wilt

| Compound | Period of treatment (days) | Volume absorbed[1] (ml.) | Disease grade relative to check |
|---|---|---|---|
| HD 3 | 2 | 75 | 0.30 |
| | 4 | 140 | 0.30 |
| | 6 | 250 | 0.35 |
| CC 1182 | 2 | 100 | 0.39 |
| | 4 | 250 | 0.44 |
| | 6 | 340 | 0.35 |

[1] Corrected for loss of solution by evaporation.

As may be seen in Table 4, there was little additional reduction in severity of disease after a two-day treatment. Studies on the chemotherapy of bean blight indicated an increased effect of treatment as the number of applications to plants was increased (12). In the experiment on Fusarium wilt, data of which are presented in Table 4, roots of plants were immersed in solutions of chemotherapeutant. Perhaps more absorption of chemotherapeutant occurs when roots are immersed in solutions than when they are exposed to chemotherapeutant under normal growing conditions. It may well be, however, that the treatment schedule can be considerably reduced in the standard assay without loss in chemotherapeutic effect, thus increasing its efficiency.

When chemotherapeutants were used in commercial greenhouses for control of Fusarium wilt of carnations, applications were made twice a week early in the experiments and once a week later on. Because these treatments effectively reduced the levels and incidence of Fusarium wilt (60), it appears that the treatment schedule in the standard assay is ample. There is no basis for the belief that, because an extended treatment is called for in the assay, chemotherapy cannot be of practical value.

## D. USE OF WASHED VERSUS UNWASHED INOCULUM

Inoculum can contain significant amounts of lycomarasmin and other toxins, formed by *Fusarium oxysporum* f. *lycopersici* in culture (21, 22, 40, 41). Since any toxins responsible for symptoms will be formed in infected plants in due course, it is desirable to eliminate any phytotoxic constituents in the inoculum which do not play a role in patho-

[1] The relation of the leaf grade, I.G. to the vascular grade used thus far in the discussion, 3VO, is discussed in Section IV, pp. 45-47.

genesis. Then any differences resulting from treatment will more accurately measure the value of chemotherapeutic treatment.

An experiment was set up, testing whether the bud cells produced in shake culture as described in the assay procedure contained toxic components which might influence the results of the assay. A batch of inoculum was grown in the usual manner. Half of it was then centrifuged, the bud cells of *Fusarium* resuspended in distilled water and recentrifuged to free them of any phytotoxic materials that might have been present. The other half was used as inoculum directly. A series of 28 tomato plants, untreated chemotherapeutically, was divided into four lots of seven plants each. Two of these lots were exposed to washed inoculum and the other two exposed to untreated inoculum. One lot of seven plants from each of these series was inoculated by the standard method of dipping injured roots into the inoculum, while the remaining plants were inoculated by injecting bud cells with a hypodermic needle into the stem at the ground line. After disease had developed, plants were graded for severity of vascular discoloration with results shown in Table 5.

Table 5. A Comparison of Severity of Fusarium Wilt, Using Two Types of Inoculum and Two Methods of Inoculation

| Type of inoculum | Severity of vascular discoloration | | |
|---|---|---|---|
| | Hypodermic inoculation | Root inoculation | Totals |
| Washed cells | 0.11 | 0.72 | 0.83 |
| Unwashed cells | 0.30 | 0.62 | 0.92 |
| Totals | 0.41 | 1.34 | |

The amount of random variation in the data of Table 5 was estimated by means of the analysis of variance. Disease levels resulting from use of washed *versus* unwashed cells as inoculum did not differ from one another. On the other hand, the two inoculation methods led to marked differences in disease level. Since it does not matter whether or not inoculum is washed before being used, no preliminary washing of bud cells is called for in preparing inoculum in the assay method.

## E. METHODS OF INOCULATING PLANTS WITH FUSARIUM

We have already seen that disease levels in untreated plants will differ significantly when different inoculation methods are used. A chemotherapy assay must utilize inoculation methods that result in uniformly severe disease levels in a population of untreated plants. Because inoculation of plants with a hypodermic needle is convenient, additional evidence was sought to determine the suitability of this inoculation method for assay purposes.

Hypodermic inoculation of *Fusarium* was compared with the standard inoculation method in a number of experiments. In a typical test,

seven compounds, some effective chemotherapeutants and some not, were applied to plants and the two methods of inoculation were compared. Each chemotherapeutic treatment was applied to 12 replicate plants, and six of these were inoculated by each method. After inoculation, all plants were uprooted, washed and repotted in clean sand in the usual way. Four weeks later, they were graded for disease severity. The mean grades of Fusarium wilt are given in Table 6.

TABLE 6. CHEMOTHERAPY OF FUSARIUM WILT USING TWO METHODS OF INOCULATING PLANTS

| Compound[1] | Concentration (ppm) | Severity of disease | |
|---|---|---|---|
| | | Hypodermic inoculation | Standard inoculation |
| CC 905 | 63 | 0.48 | 0.03 |
| CC 1182 | 16 | 0.26 | 0.00 |
| CC 1207 | 125 | 0.23 | 0.29 |
| OQP | 500 | 0.25 | 0.47 |
| PMAS | 16 | 0.55 | 0.83 |
| OQB | 500 | 0.37 | 0.74 |
| OQBo | 250 | 0.61 | 0.86 |
| Check | ..... | 0.32 | 0.97 |

[1] Compounds are reported in the table by code numbers. CC 905 is cyclopentenyl butyl dithiocarbamate; CC 1182 is 4-chloro-3,5-dimethyl phenoxy ethanol; CC 1207 is 2-norcamphane methanol. OQP is 8-quinolinol phosphate and was supplied by Darsyn Laboratories, Paterson, N. J.; PMAS is phenyl mercury acetate and was supplied by O. E. Linck Co.; OQB, 8-quinolinol benzoate, and OQBo, 8-quinolinol borate, were supplied by Mallinckrodt Chemical Works, St. Louis, Mo.

As may be seen in Table 6, in almost all cases disease levels were lower when plants were inoculated hypodermically than when inoculated by the standard method. The low level of disease in the hypodermically inoculated plants resulted from infections that were less severe and more variable within treatments than by the standard method. We may conclude that hypodermic inoculation of plants does not result in sufficiently uniform infection to justify its use in chemotherapy assays.

The behavior of plants treated with CC 905 and CC 1182 is interesting (Table 6). These compounds were effective chemotherapeutants when plants were inoculated by the standard method but failed when plants were inoculated hypodermically. Such behavior suggests that their protective effect is localized in roots and that neither the compounds themselves nor the effects they produce occur to a sufficient degree to reduce disease in aboveground parts of plants.

## F. THE INFLUENCE OF SUSCEPTIBILITY OF TOMATO VARIETIES TO FUSARIUM WILT UPON THE PERFORMANCE OF CHEMOTHERAPEUTIC TREATMENTS

An assay to determine general chemotherapeutic potency should rate treatments similarly whether the host is highly susceptible to a dis-

ease or is only moderately so. In order to measure whether the assay method fulfills this requirement, an experiment was conducted in which two varieties of tomato were used. Bonny Best, the variety employed in the assay, was used as an example of a highly susceptible host of *Fusarium*, whereas Globe was chosen as a moderately susceptible variety. Lots of five plants were treated with each of eight chemotherapeutic treatments for each variety, the entire experiment being arranged as a series of randomized blocks.

Table 7. The Performance of Some Chemotherapeutants on Bonny Best and Globe Tomatoes

| Compound[1] | Concentration (ppm) | Severity of Fusarium wilt | |
|---|---|---|---|
| | | Bonny Best | Globe |
| HD 3 | 667 | 0.05 | 0.01 |
| HD 25 | 16 | 0.37 | 0.02 |
| CC 1182 | 16 | 0.38 | 0.05 |
| CC 905 | 63 | 0.55 | 0.24 |
| CC 1207 | 125 | 0.93 | 0.51 |
| OQBo | 250 | 0.94 | 0.52 |
| PMAS | 16 | 0.80 | 0.73 |
| Check | | 0.72 | 0.45 |

[1] The chemistry of these compounds, with the exception of HD 3 and HD 25, is given in the footnote to Table 6. HD 3 is *n*-octadecyl trimethyl ammonium pentachlorophenate and HD 25 is N-(4-nitrophenyl)-3,4-dichlorobenzene sulfonanilide.

Table 7, which summarizes the results of the experiment, indicates that the requirements outlined above are indeed fulfilled. As might be expected, the disease levels on Globe were always lower than on Bonny Best for the same treatment, because of the genetic resistance of the former, but the different treatments were ranked in strikingly similar order for each variety.

Genetic resistance to disease may depend upon the presence of specific compounds in the plant. In repeated chemotherapy assays a compound can be effective on a highly susceptible variety but be considerably less effective on a resistant variety. When this happens, it may be worthwhile to study the mode of chemotherapeutic action of this compound and its derivatives with a view to accounting for the mechanism of resistance of the variety that does not respond to treatment. Such an approach may be profitable to both the plant pathologist and the geneticist in establishing criteria for resistance that are rapid and quantitative.

## G. NATURE OF THE DOSAGE-RESPONSE CURVE OF CHEMOTHERAPEUTANTS

A study of the nature of the dosage-response curve for any effective compound is critical to this method of assessing chemotherapeutants, to the concept of chemotherapy generally, and to its application to problems of controlling plant diseases under field conditions. If the effectiveness of a compound decreases as its concentration does, it can be applied at the maximum non-phytotoxic concentration only. If there is little

effect of treatment at this concentration, there will be even less at a lower one. More important, one establishes a cause and effect relationship between the compound applied and the decrease in severity of disease. In the presence of such a relationship, the effect can hardly be considered an artificial or chance result. Finally, the nature of the dosage-response curve indicates what the effective chemotherapeutic concentration is. This concentration, relative to the maximum non-phytotoxic concentration, determines whether control of disease may be expected in practice and whether treatment is economically feasible.

The relation of the concentration of chemotherapeutant to the level of disease resulting from treatment has been measured many times in this laboratory. Data on three compounds will suffice to illustrate that chemotherapeutants generally have linear dosage-response curves when data are suitably transformed. HD 160 (sodium salt of 2-carboxymethyl mercaptobenzothiazole), HD 3 (*n*-octadecyl trimethyl ammonium pentachlorophenate) and CC 910 (2-[4-morpholinyl] ethyl phenyl ketone) were applied in graded concentrations to different lots of plants and disease grades were obtained for each compound at each concentration. In Figures 3 and 4 the concentration at which the compound under test was applied has been plotted against the severity of disease which resulted from treatment. These data are plotted in the two figures on the basis of two assumptions: (a) that the response of the plant, percentagewise, is directly proportional to the logarithm of the concentration of chemotherapeutant applied to the plant (Figure 3), or (b) that resistance to disease is normally distributed among plants and that there is a decreasing return per unit increase in concentration of chemotherapeutant (Figure 4).

Evidently the latter of these two assumptions is the more nearly correct, since a log-probit plot of concentration of chemotherapeutant against severity of disease results in a straight line relationship. Therefore, one is justified in measuring the chemotherapeutic potency of compounds at the maximal non-phytotoxic concentration and in assuming that, if no chemotherapeutic activity is evident under these conditions, there is little chance of observing activity at a lower concentration. A note of caution should be sounded at this point, however. The investigator will be quick to note that, when a compound is phytotoxic, the symptoms of phytotoxicity may be confused with symptoms of disease (Figure 9) and the value of the compound as a chemotherapeutant may be improperly assessed at high concentrations. When compounds have been applied at phytotoxic concentrations, therefore, their chemotherapeutic potency must be measured at lower concentrations, where phytotoxic symptoms are not evident.

The relation between the logarithm of concentration at which a chemotherapeutant is applied and the probit of disease severity is one of direct proportion. One may therefore compare chemotherapeutants by the same criteria as are used in evaluating fungitoxicity, *viz.*, the ED 50 and slope of the dosage-response curve.

When protectant fungicides are assayed in the laboratory, slope of the dosage-response curve is determined primarily by the change in the

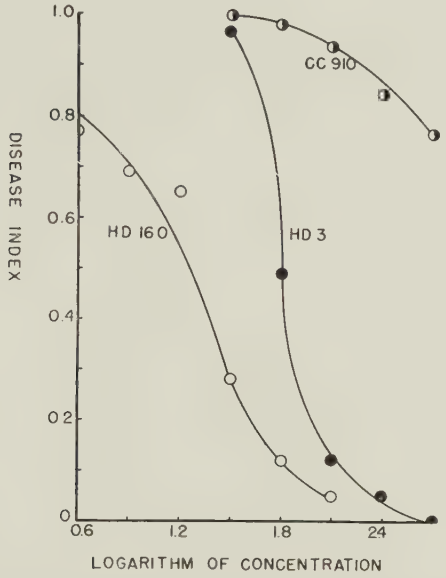


**Figure 3. Sigmoid dosage-response curve of three chemotherapeutants when logarithm of concentration applied to plant is plotted against the chemotherapeutic ratio. (The chemotherapeutic ratio is obtained by dividing the mean severity of Fusarium wilt in treated plots receiving a given treatment by that in check plots.) HD 160 is the sodium salt of 2-carboxymethyl mercaptobenzothiazole, HD 3 is n-octadecyl trimethyl ammonium pentachlorophenate, and CC 910 in 2-(4-morpholinyl ethyl phenyl ketone).**

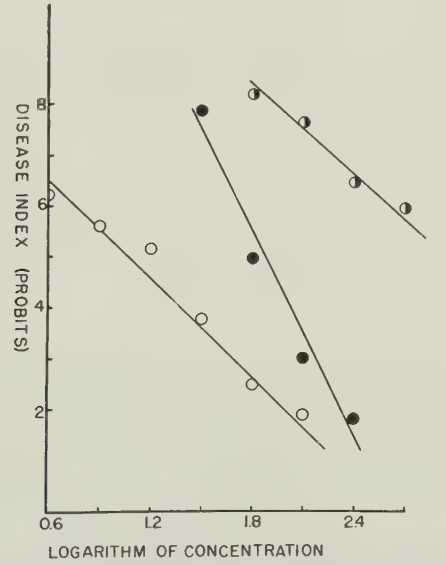


**Figure 4. Dosage-response curve for the data plotted in Figure 3 when logarithm of concentration used in treatment is plotted against the chemotherapeutic ratio expressed in probits.**

number of susceptible spores among a population for unit change in log-dosage of fungicide. In field assays of protectant fungicides, the likelihood of failure of the pathogen to infect the host is the resultant probability of all the events that may cause spores to die, and of these a lethal dosage of fungicide is only one. Hence, the slope of the dosage-response curve in the field is the change in this resultant probability with dosage of fungicide.

The dosage-response curve for a chemotherapeutant, like that of the protectant fungicide assayed under field conditions, is determined by all the events that may cause the infection process to fail. Chemotherapeutants, however, will be active against the germ tube or established mycelium already inside of the host. In brief, the dosage-response curve for a chemotherapeutant indicates the distribution of susceptibility of the disease to dosage of chemotherapeutant, expressed logarithmically.

## H. PHYTOTOXICITY OF COMPOUNDS AND THE SUSCEPTIBILITY OF TOMATOES TO FUSARIUM WILT

Any compound may be phytotoxic if applied to plants at sufficiently high concentration. In the assay procedure an effort is made to determine the concentration below which phytotoxicity is not serious. Compounds are then applied at or below this concentration. The estimate, however, is frequently in error and when compounds are applied at the indicated concentrations, they may be seriously phytotoxic. In such cases the compound is retested at lower concentration before a judgment is reached concerning its chemotherapeutic potency. The estimate of chemotherapeutic potency may be grossly in error when compounds are assayed at concentrations which lead to conspicuous phytotoxicity in the test plant.

In a chemotherapy assay, results must be obtained on plants which are growing with comparable vigor. If plants are not equal in vigor, they may vary in their susceptibility to disease, apart from chemotherapeutic effects. The hard, woody tomato plant will differ in susceptibility from the succulent one, apart from the effects of the compound *per se* on susceptibility of the plant or the pathogen. A number of preliminary assays of compounds have been made at concentrations producing severe to moderate phytotoxicity. When these same compounds were retested at concentrations that were not phytotoxic, some were more effective as chemotherapeutants while others were less so. Various kinds of chemical injury evidently led to variation in susceptibility of tomatoes to Fusarium wilt.

We have already seen that, when assayed in dosage series at non-phytotoxic levels, a compound causes disease severity to vary as a straight line function of concentration when data are suitably transformed. When the chemotherapeutic potency of a compound appears to increase as it is applied to plants in more dilute solution, the results[1] of the assay may be reasonably questioned.

[1] There is every reason to expect that on rare occasions chemotherapeutants will be found which, in dilution series, generate curvilinear dosage-response curves, just as protectant fungicides do. Tetramethyl thiuram disulfide is an example of a fungicide which, in spore germination assays, has a curvilinear dosage-response curve (26).

## I. THE INACTIVATION OF CHEMOTHERAPEUTANTS IN SOIL VERSUS SAND

As a growing medium, sand is more likely than soil to give results that can be reproduced from one laboratory to another, because it will not vary so widely in composition as soil does. Soils vary more than sand in base exchange capacity and in the populations of microorganisms they contain. Base exchange and microbial action may result in adsorption and breakdown of chemotherapeutants to such an extent that practically none of the chemotherapeutant may enter a plant when it is applied to a soil.

The behavior of a pair of compounds will illustrate the extent to which chemotherapeutants may be reduced in potency in the soil as compared with their activity in sand. Two compounds, CC 1182 and HD 25, both promising chemotherapeutants for Fusarium wilt, were applied to tomato plants at a concentration of 32 ppm. Each was applied to five replicate plants grown in sand and to five plants grown in soil. Comparable numbers of plants were left as checks in sand and soil. Results of the experiment, expressed as the disease index in treated plants relative to that in the corresponding checks, are shown in Figure 5. It is apparent that CC 1182 is equally potent in sand and soil, while there is a very material reduction in potency of HD 25 when applied to a Cheshire fine sandy loam as compared with sand.

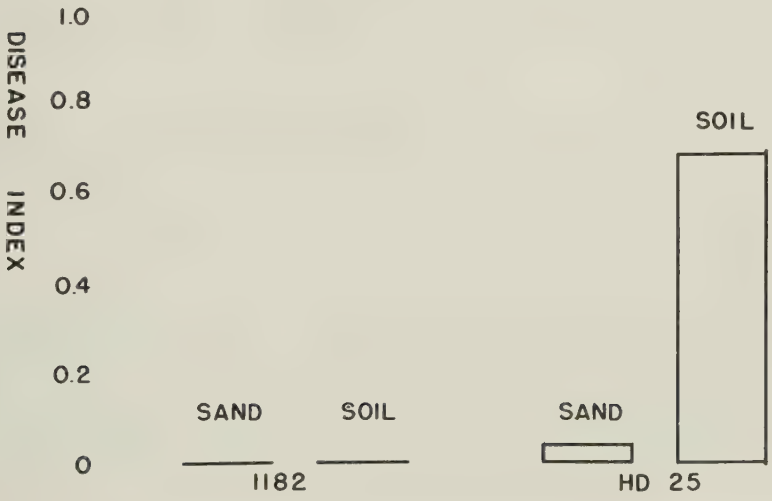


**Figure 5. The potency of two compounds when applied as chemotherapeutants to roots of plants growing in sand and in soil.**

An assay method should not necessarily eliminate at the outset compounds that are inactivated in soils. The objective of assessing chemo-

therapeutic potency may be immediately practical or it may be an interest in relations of chemical structure to activity and in studies on how chemotherapeutants act in plants. In studies of the latter type, the relative performance of compounds without regard to soil inactivation becomes important, and each factor influencing performance must be appreciated and evaluated.

## J. DURATION OF THE EFFECT OF TREATMENT WITH CHEMOTHERAPEUTANTS

So far as Fusarium wilt of tomato is concerned, there is no evidence with any compound yet encountered indicating that plants once treated are rendered permanently resistant to disease. Plants appear to be made significantly more resistant to disease for a time, but the effect of treatment is, in general, short lived. Two types of experimental evidence can be presented which bear upon this point.

The first is based upon the change in disease grade with time among a series of treated plants when severity of disease is measured serially. In one experiment eight treatments were applied in the usual manner and the severity of disease was measured by determining leaf grades[1] 15 and 21 days after plants were inoculated. The mean leaf grades for six replicate plants per treatment are presented for the two observations in Table 8.

In all cases but one, the disease grade increased during the six days that elasped between the first and second readings. Plants treated with CC 1182 were still free of disease at the end of the second reading and the disease grade remained unchanged in this case. But with the exception of CC 1182, this indicates that the effect of treatment gradually disappears.

Chemotherapeutants are evaluated by rating the severity of disease at the end of a given period of incubation. Good chemotherapeutants will be evident because of the high level of protection or cure which they bring about. The disease level at inoculation time, $t_o$, is obviously zero, and at time, t, a treatment is assigned grade x. Therefore, the chemotherapeutant is evaluated, not in terms of the grade for disease severity, but rather in terms of the change in disease grade, $\triangle x$, which takes place within a given time period, $\triangle t$. By the same reasoning,

[1] The plant is dissected in obtaining a disease rating based on vascular discoloration. To obtain serial readings of disease severity on a group of plants, leaves were graded for the severity of wilting symptoms on a scale from 0 to 4. The relation of the leaf grading system to the grading system based on vascular discoloration (3VO) which has been used in previous discussions is discussed on pages 45-47.

TABLE 8. THE CHANGE IN LEAF GRADES OF CHEMOTHERAPEUTICALLY TREATED PLANTS WITH TIME

| Compound[1] | Concentration (ppm) | Leaf grades (LG) (time after inoculation) | | Change in leaf grade per day | |
|---|---|---|---|---|---|
| | | 15 days | 21 days | First 15 days | Last 6 days |
| CC 1182 | 16 | 0.00 | 0.00 | 0.00 | 0.00 |
| OQP | 500 | 1.62 | 1.80 | 0.11 | 0.03 |
| PMAS | 16 | 1.68 | 2.30 | 0.11 | 0.10 |
| CC 1207 | 125 | 0.14 | 0.72 | 0.01 | 0.10 |
| CC 905 | 63 | 0.26 | 0.38 | 0.02 | 0.02 |
| OQB | 500 | 1.49 | 2.33 | 0.10 | 0.14 |
| OQBo | 250 | 1.82 | 2.70 | 0.12 | 0.15 |
| Check | ----- | 2.53 | 3.36 | 0.17 | 0.14 |

[1] See footnote to Table 6.

one can evaluate the remaining potency of a treatment some time after it was applied by the change in grade since the last disease reading was taken. In this way, one may compare the relative stability of chemotherapeutic treatments. This principle has been applied in Table 8. In column 5 is given the increase in disease grade per day during the first 15 days and in column 6 the increase in grade per day is given from the 15th through the 21st day. A comparison of these values for the several treatments indicates that CC 1182 and CC 905 were still effective after 21 days whereas CC 1207 had lost its effectiveness by that time.

A second line of experimental evidence shows that the chemotherapeutic effect wears off in time and the plant gradually becomes invaded by the pathogen. Even though the compound CC 1182 has proven to be a reasonably effective chemotherapeutant in many experiments, plants treated with it eventually succumb to Fusarium wilt if treatment is not repeated after inoculation. The gradual decline in plants from wilt after treatment with CC 1182 is shown in Figure 6. Data on which this figure is based were drawn from 10 experiments extending over a period of three years, and the time given is that which has elapsed between the day plants were inoculated and the one on which they were read for severity of disease. To make the data more comparable from one experiment to another, the 3VO grades in plants treated with CC 1182 have been divided by grades for untreated control plants in the same experiment, read at the same time. It will be seen that, after a 40 day period, the disease levels in the treated plants were rapidly increasing and that they would eventually approach the levels in the checks.

## K. EVIDENCE THAT COMPOUNDS ARE ABSORBED BY THE PLANT AND THAT THE EFFECTS ARE INTERNAL, i. e., CHEMOTHERAPEUTIC

The assay procedure, given in Section II, has been designed so that if plants can absorb chemotherapeutant, they will do so. Similarly, the assay has been designed so that compounds whose activity depends upon killing of the pathogen outside of the plant will not be considered to be chemotherapeutants. Thus, compounds are applied in solution or sus-

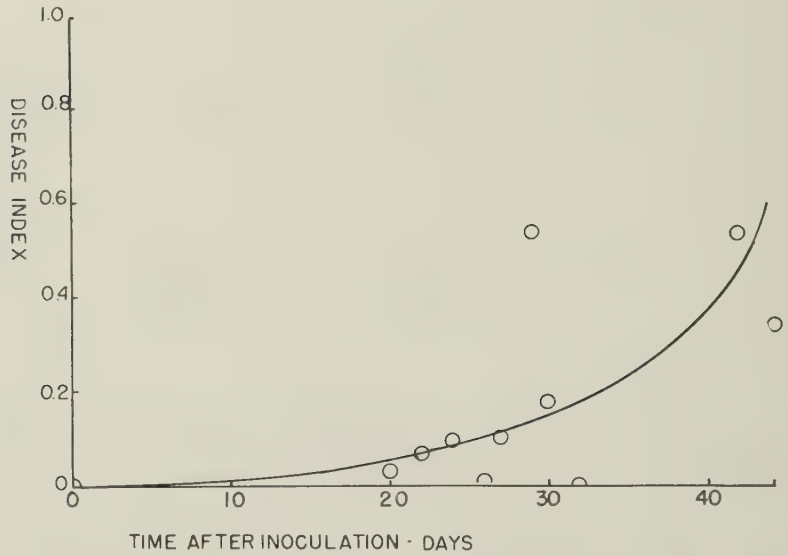


**Figure 6. The decline in chemotherapeutic effectiveness with time. Time is measured in days after the last application. The chemotherapeutant was 4-chloro-3,5-dimethyl phenoxy ethanol (CC 1182).**

pension to the roots of plants. The roots of the plant are not inoculated until they are first washed and then injured. The washing is intended to remove any chemotherapeutant still in solution or suspension on the root surface. Injury exposes freshly wounded surfaces of the plant to inoculum, surfaces that have not previously been exposed externally to the chemotherapeutant. If infection then occurs, it does so despite compounds that may be present inside the root.

There is abundant evidence that many of the compounds applied do enter plants. Such compounds as CC 1182 have a formative effect on tomatoes and the formative effect is clearly evident on leaves that were on the plant or were expanding when application was being made. Other compounds, such as CC 1207, have a characteristic odor, and the foliage of plants treated with this compound smells similarly.

It has been suggested repeatedly that compounds which are fungicidal may act merely by killing the pathogen before it can enter the plant and establish itself and that the effect of such compounds is from an adsorbed layer of fungicide over the surface of the root system. While this might be true if the plant were uninjured during the inoculation process, it is difficult to see how a plant could be protected if a portion of the adsorbed layer of toxicant were removed through root injury and the roots then exposed to inoculum, as is done regularly. Moreover, in many cases compounds having excellent chemotherapeutic properties have no demonstrable fungitoxicity at the concentrations used

in assays. Frequently chemotherapeutants would have to be used at concentrations lethal to the plant if they were to be employed at fungitoxic (ED 50) concentrations.

The results of a single experiment will indicate that more is involved than mere adsorption of compound by root surfaces. 4-Chloro-3,5-dimethyl phenoxy ethanol (CC 1182) is mildly fungitoxic at concentrations which are also chemotherapeutic. Concentrations of 16 ppm of this compound were placed in five beakers. Two-month-old tomato plants, whose roots had been washed free of sand, were placed in these solutions, five to a beaker. The five lots of plants were then removed according to the following schedule: (a) removed immediately, roots washed, injured, inoculated and planted; (b) removed immediately, roots injured without washing, inoculated and planted; (c, d and e) plants allowed to stand in solutions of chemotherapeutant for 24, 48 and 96 hours, respectively, after which roots were washed, injured, inoculated and planted.

Three weeks after being inoculated, plants were graded for severity of disease in terms of the number of plants that showed vascular discoloration in the stem. These results, together with the volume of solution absorbed, are shown in Table 9.

Table 9. Relation of Time of Chemotherapeutic Treatment to Number of Plants Contracting Fusarium Wilt

| Treatment[1] | Volume absorbed (ml.) | No. of plants showing vascular discoloration |
|---|---|---|
| a | 0 | 5 |
| b | 0 | 5 |
| c | 65 | 5 |
| d | 119 | 5 |
| e | 125 | 0 |

[1] See second paragraph above for details of treatments.

Were simple adsorption of compound involved, a difference in response of plants would surely be expected by the time plants had been standing in solution for 24 hours, since by then adsorptive surfaces would be saturated. Were mere volume of chemotherapeutant absorbed the controlling factor, there should be no essential difference in response of the groups of plants subjected to treatments d and e (Table 9). Evidently time is required for absorption of a chemotherapeutic dose by the plant and for its adequate distribution. Alternatively, time is required for changes in host metabolism to occur which increase resistance of the plant to Fusarium wilt.

## IV. Grading Methods, Their Evaluation and Interrelations

It is possible to grade for the severity of Fusarium wilt in tomatoes by any of several methods. Since *Fusarium* causes wilting of leaves and also produces discoloration of vascular bundles in stem and petiole, the

disease may be graded in terms of leaf wilting, discoloration of petiole bundles or of bundles in the stem. In addition, vascular discoloration may be graded with respect to its presence or absence, or with respect to intensity. The investigator may well ask which single grading system is best suited to discriminating differences in level of Fusarium wilt and which method best appraises treatment effects. It is also important to know which grading methods are least affected by season and by positional effects of the plant in the greenhouse bench.

## A. EVALUATION OF SEVERAL POSSIBLE GRADING METHODS

The first portion of the present section contains the results of a study of several grading methods, aimed at answering such questions. It will be necessary, however, to describe in detail the several grading methods that have been used.

### 1. Description of Grading Systems

#### a. LEAF GRADES

The severity of disease in plants may be graded in terms of the wilting symptoms on leaves. Leaf grades were set up in these studies, based upon the following conventions:

Grade 0 — no disease symptoms.
Grade 1 — slight symptoms, including epinasty and/or slight yellowing of the terminal leaflet.
Grade 2 — moderate symptoms, including as much as 50 per cent of the leaflet area yellowed or flaccid, but no necrosis.
Grade 3 — severe symptoms, involving complete yellowing and/or flaccidity but necrosis not complete.
Grade 4 — leaf missing or completely nonfunctional.

Each plant is read leaf by leaf from the bottom to the top, and data are recorded in this way. The disease grade for the plant is the sum of the individual leaf grades, divided by the number of leaves. The leaf grading system (LG) is based upon the behavior of all leaves on the plant.

This grading method has one disadvantage: it ignores defoliation of the oldest leaves brought on by transplanting and handling the plants. Actually the two oldest leaves are frequently (but not invariably) dropped even from uninoculated check plants as a result of these operations and from aging. For this reason the leaf grade is never 0. Ideally the leaf grade should be corrected for this effect so that uninoculated check plants and those treated with effective chemotherapeutants would have a grade of 0. The grade for uninoculated check plants (which have received root injury but no chemical treatment) varies from one experiment to another and so it is not possible to correct for the dropping of leaves by healthy plants by deducting a constant from the leaf grade which is valid over all experiments.

In an effort to correct this difficulty a second leaf grade was investigated. This grade (hereafter referred to as MLG for modified leaf grade) was obtained in the same way as the leaf grade, but differed in that the response of the two bottom true leaves on the plant was ignored.

### b. VASCULAR DISCOLORATION IN THE STEM

Vascular discoloration in the stem may be assessed on the basis of whether all six bundles are discolored or attention may be confined to the discoloration in the three largest ones. Moreover, one may attempt to grade intensity of discoloration in each bundle or simply grade a bundle as discolored or not. At one time it was a standard procedure to grade intensity of discoloration in stems according to the following conventions:

Grade 0 — no discoloration
Grade 1 — very slight olive-brown discoloration
Grade 2 — moderate discoloration
Grade 3 — severe, chocolate-brown discoloration

Bundles were graded according to this system at each internode. The mean grade for the plot was obtained by summing these individual grades for all bundles and all internodes and dividing by the total number of bundles observed.

It will be evident that vascular discoloration in the stem may be graded in four possible ways and that these grading systems will differ in their maximum values, according to whether or not an attempt is made to score for the intensity of discoloration. The following grading systems have all been used.

| *Grading system* | *Symbol*[1] | *Range of values* |
|---|---|---|
| 6 bundles graded for intensity of discoloration | 6VC | 0 - 3 |
| 6 bundles graded for presence of discoloration | 6VO | 0 - 1 |
| 3 bundles graded for intensity of discoloration | 3VC | 0 - 3 |
| 3 bundles graded for presence of discoloration | 3VO | 0 - 1 |

### c. VASCULAR DISCOLORATION IN THE PETIOLES

Finally, discoloration is produced in the vascular bundles of the petiole. The petiole contains five bundles, a median one and two pairs of lateral bundles. The outermost pair is so small as rarely to be visible to the naked eye and was ignored in the grading system. Again, one may grade for intensity of discoloration, using the conventions given for discoloration in the stem. This grade, referred to hereafter as the PC grade, may range from 0 for the healthy plant to 3 for the plant that is severely diseased. In contrast one may grade bundles only as discolored or not. This grade, the PO grade, varies from 0 in the healthy plant to 1 in the severely diseased one.

[1] When O occurs in a grading symbol, it should be read as zero, e. g., 3VO is read three vee zero. The 3VO indicates that three vascular bundles (in the stem) were read but that zero weight was given to intensity of discoloration. C stands for color in the corresponding grading system and indicates that intensity of color was scored.

### d. SUMMARY OF THE GRADING SYSTEMS EMPLOYED

To aid the reader in keeping in mind the several grading systems which will be discussed in the following pages, Table 10 is presented. It summarizes eight grading systems in terms of the symbol by which each is designated, and states also how the grade is obtained in each case and the range.

TABLE 10. SUMMARY OF THE GRADING SYSTEMS STUDIED FOR EVALUATING THE SEVERITY OF FUSARIUM WILT OF TOMATO

| Symbol[1] | Method of obtaining the grade | Range of values |
|---|---|---|
| LG | All leaves graded for wilt on scale from 0 to 4 | 0 - 4 |
| MLG | Bottom 2 leaves ignored; otherwise identical with LG | 0 - 4 |
| PC | 3 petiole bundles scored for intensity of discoloration | 0 - 3 |
| PO | 3 petiole bundles scored for presence of discoloration | 0 - 1 |
| 6VC | 6 stem bundles graded for intensity of discoloration | 0 - 3 |
| 6VO | 6 stem bundles scored for presence of discoloration | 0 - 1 |
| 3VC | 3 large stem bundles graded for intensity of discoloration | 0 - 3 |
| 3VO | 3 large stem bundles scored for presence of discoloration | 0 - 1 |

[1] See footnote, page 31.

## 2. Comparison of Grading Systems at a Single Time

To determine which grading system gave the most information concerning the severity of disease, plants in the experiment described on page 14 were graded by all eight methods. Analyses of variance were then run for each grading method, all being made in the same manner. Three criteria were employed in evaluating the several grading methods: (a) the coefficient of variation measured random variation for the several grading systems in comparable units despite their varying ranges, (b) the ability of a grading method to assess treatment effects was measured through the variance ratio for treatments, and (c) the extent to which a grading method was affected by positional effects was evaluated through the variance ratio for blocks.

On the basis of previous assays of chemotherapeutic potency, some of the compounds used in this experiment were chosen because they were highly effective, some moderately so and some quite ineffective. Because the treatments were selected in this manner, they collectively elicited responses in disease grade that covered the full range of possible values for each grading system. Therefore, comparisons of grading methods on the basis of the variance ratio for treatments are considered to be valid.

Another matter was also evaluated in this study, namely, whether it is desirable to transform the data in such fashion that the variance is equalized over all portions of the response range. The analysis of variance assumes that data are expressed in this manner. For a given grading system, plots of the range of observations within a treatment against the mean indicated that variation within treatments was a minimum

when plants were either only slightly diseased or when they were severely diseased and was a maximum in the mid-region of the response range. To equalize such variation, raw data for three interesting grading systems were transformed to equivalent angles (53) and analyses of variance were run upon them also. By using the same criteria as were used in the analysis of untransformed disease indices, it was possible to appraise the value of transformation.

For the latter study the two grading methods were selected that best met the criteria given above, the leaf grade and 3VO (three vee zero) systems and also one grading method that met these criteria but poorly, the PO grading system. Before transforming the leaf grades, it was necessary to divide all values by 4, the maximum value possible, so that the range of response was changed from 0 to 1.

The results of all these analyses are presented in Table 11, which permits a direct comparison of the several grading systems.

TABLE 11. AN EVALUATION OF EIGHT GRADING SYSTEMS FOR ASSESSING THE SEVERITY OF FUSARIUM WILT

| Grading method[1] | Coefficient of variation | Variance ratio: F | |
|---|---|---|---|
| | | Treatments (20 x 78 DF) | Blocks (4 x 78 DF) |
| Grades analyzed directly | | | |
| LG | 0.310 | 15.77** | 11.39** |
| MLG | 0.413 | 13.86** | 11.53** |
| PC | 0.622 | 7.90** | 3.59** |
| PO | 0.561 | 10.84** | 5.62** |
| 6VC | 0.569 | 10.48** | 2.12 n.s. |
| 6VO | 0.471 | 13.86** | 6.28** |
| 3VC | 0.462 | 13.15** | 4.93** |
| 3VO | 0.362 | 19.06** | 3.46* |
| Grades transformed to equivalent angles | | | |
| LG | 0.258 | 13.89** | 11.37** |
| PO | 0.445 | 13.99** | 5.27** |
| 3VO | 0.337 | 15.68** | 4.08** |

[1] See Table 10 for a summary of how these grading systems differ from one another.
* Effects significant at odds 1:20.
** Effects significant at odds of 1:100.
n.s. Effects not significant at odds of 1:20.

Table 11 reports a number of pertinent comparisons of the grades. In the first place the coefficient of variation is lower for a given grade if it is first transformed to equivalent angles than if the data are analyzed in raw form. However, increased ability to discriminate treatment differences[1] appears to be negligible because the variance ratios for treatments are not increased in the case of the two most useful grades: 3VO and LG.

[1] Finney (16), who has carefully studied the value of transforming data with the same objective, has concluded that "the practical conclusions from an assay will seldom be seriously affected even by violent changes in the response metameter; the transformations $y^{\circ} = y^{2}$ and $y^{\circ} = y^{-1}$ are sharply contrasted, yet in two examples they produce substantially the same conclusions on potency." Finney emphasizes that it is practical conclusions on potency which are substantially unchanged. In theoretical studies of some types, transforming disease grades will probably be distinctly worthwhile.

Grading methods may also be compared when data are not transformed to equivalent angles. Restricting our attention to grading methods based upon vascular discoloration, one notes that the coefficient of variation is reduced when intensity of discoloration is ignored (Table 11). This results in a material increase in the variance ratio for treatments — a real advantage.

A comparison of all grading systems on the basis of the variance ratio for treatments yields additional information. One sees at a glance that the petiole grades and the 6VC grade do not designate as significant, treatments with marginal effects so well as the other grading systems do. The two "best" grading systems, by this criterion, are the leaf and 3VO grades.

Finally, one may judge the sensitivity of the grading systems to positional effects by the variance ratio for blocks. Quite evidently the leaf grades and modified leaf grades are very sensitive to positional effects; so also is the 6VO grade. All things considered, the leaf and 3VO grades appear to be most useful and are also the easiest to read. If a single grading system is to be chosen, the 3VO grade best meets the requirements previously set up.

How does it happen that a grading system based on mere presence of discoloration is more precise than one in which intensity of discoloration is graded? Scoring the differences in color intensity in vascular bundles is likely to be difficult and some variation appears to be introduced when the grading system requires that these discriminations be made. The data suggest that presence of discoloration is a less variable measure of the severity of Fusarium wilt than intensity of discoloration is.

We may equally well inquire how it happens that a grade based upon the three largest vascular bundles is more precise than one based on all vascular bundles. It is frequently very difficult to distinguish the presence of discoloration in very small bundles in the stem. When the operator attempts to grade the small bundles, he is likely to become confused. Sometimes he will grade them lower than large ones simply because they are smaller; at other times when color is intense he will grade them as high as he grades large bundles. Thus, he tends to carry a more variable score for the small bundles than for the large ones. In turn, this tends to score the stem as a whole more variably. Thus, valuable information is lost in assessing the value of treatments.

With the 3VO grade the differences between plants are more closely related to treatment differences and less to positional effects than with any other grade. In contrast, the leaf grade, while it distinguishes treatment differences readily enough, is more influenced by positional effects than the vascular grade is. As we shall see, the leaf grade is also more susceptible to seasonal effects than is the vascular grade.

These conclusions have been reached on the basis of attributes of grades within a single experiment. To what extent are these conclusions confirmed in other experiments? Most crucial is the decision that the 3VO grade more critically measures treatment effects than does the 6VC

grade. Intuitively an investigator would be inclined to the view that the 6VC grade, being based on more kinds of information, would lead to better differentiation of treatment effects. A comparison of the 6VC grade with the 3VO grade was made for six additional experiments, which were conducted during a period of about two years. In all cases the 3VO grade was at least as good as the 6VC grade if not superior to it. In view of the greater simplicity of making observations for the 3VO grade than for the 6VC grade, the advantage in quantitative character is particularly fortunate!

We must conclude from this analysis that, if a single grade is to be chosen, the 3VO grade is the preferred one. There are a number of kinds of experiments where this single grade will give all of the information needed; experiments of this sort will deal with whether or not a treatment is effective. However, if relations of chemical structure to activity are under investigation, it might be thought worthwhile to grade plants by the LG and 3VO grades together, especially when phytotoxicity studies are involved. In this connection, an analysis in which the leaf and 3VO grades have been combined is discussed on page 46.

In the experiment described on page 14 and analyzed in Table 11, 11 treatments reduced Fusarium wilt to a highly significant degree ($P=0.01$) when the 3VO grade was used (Table 2). Using the least significant mean difference between treated and check plots, one may make a similar evaluation of treatment effects for each of the other grading systems. The number of treatments causing highly significant reduction in disease levels is shown in Table 12.

Table 12. The Number of Treatments Significantly Reducing the Severity of Fusarium Wilt for Eight Methods of Grading Disease

| Grading method | Number of effective treatments[1] | |
|---|---|---|
| | Untransformed data | Transformed data |
| LG | 10 | 8 |
| MLG | 10 | |
| PC | 10 | |
| PO | 10 | 10 |
| 6VC | 10 | |
| 6VO | 9 | |
| 3VC | 10 | |
| 3VO | 11 | 11 |

[1] Treatments were judged effective when the treatment mean exceeded the least significant mean difference from the mean for check plots with high probability ($P=0.01$) of significance.

Again the 3VO grading system offers advantages over all the others, but no great advantage results from transforming data to equivalent angles before analysis. In fact, when leaf grades are transformed, it appears that fewer treatments are judged to differ significantly from the check than when data are not transformed.

Finally, it is worthwhile to consider whether the several grading systems rank treatments in approximately the same order. A detailed

study of the ranking order of treatments for all grading systems shows that the order is not greatly affected, whatever grading method is used. By way of summarizing this study, Table 13 presents the ranking order for treatment means by the 3VO grading system, together with the mean ranking order of treatment means for all eight grading systems, using untransformed data. The ranking order is strikingly similar in both columns of Table 13.

TABLE 13. THE RANKING ORDER OF TREATMENTS FOR THE 3VO GRADING SYSTEM AND AVERAGED RANKING ORDER FOR ALL 8 SYSTEMS

| 3VO | All grading systems |
|---|---|
| J | I |
| I | J |
| C | C |
| P,T[1] | T |
| K | K,P[1] |
| N | A |
| A | N |
| E | E |
| D | D |
| G | G |
| H | H |
| R | F |
| F,M,S[1] | M,R[1] |
| Q | Q |
| O | S |
| U | B |
| B | U |
| L | O |
| | L |

[1] Treatments were tied for ranking order.

The similarity with which the various grading systems evaluate treatment effects may also be studied through evaluating correlation coefficients. Data on this approach are presented subsequently (Section IV, B, Interrelations of the grading systems, page 39).

We may conclude from this study that transformation of data[1] is not worthwhile and that, of all the grading systems studied, the 3VO system, presented under the assay method, is the most useful. It has been shown that treatments will be ranked in approximately the same order by any method, but that when the 3VO grading method is used, one obtains best differentiation of treatment effects.

### 3. Relative Value of Grading Systems at Different Times

How consistent are the grading systems over the course of the winter and summer seasons, for good treatments as well as for poor ones? To answer this question, two compounds were used: 4-chloro-3,5-dimethyl phenoxy ethanol (CC 1182) at a dosage of 15.6 ppm and 2-norcamphane methanol (CC 1207) at a dosage of 125 ppm. The first of these was selected as representative of a useful treatment chemotherapeu-

[1] See footnote on page 33.

tically. The second, although it was effective in original tests, was quite inadequate when a second sample was supplied and it was chosen to represent an ineffective treatment. An inoculated control served as the third treatment in this comparison. This experiment ran over a period of a year and a half, and consisted of four comparisons of each treatment: two comparisons were made during winter months and two during summer months. These comparisons were set up as randomized blocks replicated five times in each operation.

Because certain of the grading systems proved not to be of further interest in the analysis presented in the previous section, the two grading methods based on discoloration of petiole bundles were not used in the present study. Two other grading systems were chosen as representative of "uninteresting" grading systems: the grading methods based on six vascular bundles in the stem. The other four grades: LG, MLG, 3VC and 3VO were analyzed as representative of grading systems which provide interesting information on chemotherapeutic treatments.

When all plants had been graded, data were assembled and analyzed for variance with the results presented in Table 14. These provide information on several attributes of the six grading systems under analysis.

TABLE 14. RELATIVE VALUE OF SIX GRADING METHODS AT DIFFERENT TIMES IN EVALUATING CHEMOTHERAPEUTIC TREATMENTS

| Source of variance | DF | Mean square for grading systems | | | | | |
|---|---|---|---|---|---|---|---|
| | | LG | MLG | 6VC | 6VO | 3VC | 3VO |
| Total | 59 | | | | | | |
| Blocks | 19 | 0.6948 | 0.9084 | 0.3231 | 0.0631 | 0.2754 | 0.0543 |
| Times | 3 | 3.0469 | 4.1074 | 0.8690 | 0.1157 | 0.2915 | 0.1110 |
| Seasons | 1 | 2.1736 | 2.5792 | 1.3863 | 0.2774 | 0.5453 | 0.2124 |
| Within summer | 1 | 5.0676 | 5.2508 | 0.1717 | 0.0374 | 0.0791 | 0.1116 |
| Within winter | 1 | 1.9814 | 4.4931 | 1.0491 | 0.0077 | 0.2505 | 0.0026 |
| Blocks in times | 16 | 0.2538 | 0.3086 | 0.2351 | 0.0382 | 0.2724 | 0.0437 |
| Treatments | 2 | 37.1757 | 39.4398 | 14.6398 | 2.0326 | 16.7815 | 2.8844 |
| 1182 vs. 1207 & Ck. | 1 | 74.2613 | 78.7968 | 24.5083 | 5.0471 | 33.5598 | 5.7597 |
| 1207 vs. Check | 1 | 0.0902 | 0.0827 | 0.6051 | 0.0181 | 0.0032 | 0.0046 |
| Treatments x Blocks | 38 | 0.3777 | 0.4364 | 0.2933 | 0.0763 | 0.3022 | 0.0561 |
| Treatments x Times | 6 | 0.9318 | 1.2831 | 0.6642 | 0.1149 | 0.5000 | 0.1019 |
| Treatments x Blocks in times | 32 | 0.2739 | 0.2776 | 0.2231 | 0.0690 | 0.2651 | 0.0476 |

### a. HOW WELL ARE TREATMENT DIFFERENCES DISCRIMINATED IN ALL SEASONS?

This may be answered by calculating the variance ratio obtained when the mean square variance for treatments is divided by the error term for treatments x time in the case of each of the six grades. These results are presented in Table 15.

Table 15. The Variance Ratio: Treatments Divided by Treatments x Times Error for Six Grading Systems

| Grading System | Variance Ratio |
|---|---|
| LG | 39.90** |
| MLG | 30.75** |
| 6VC | 22.05** |
| 6VO | 17.70** |
| 3VC | 33.58** |
| 3VO | 28.35** |

** Variance ratio highly significant (P = 0.01).

On this basis the grades on six vascular bundles are of distinctly less value than the other grades under consideration. Moreover, there is little choice between the two grading systems based on three vascular bundles, but the leaf grade (LG) differentiates treatment effects to the greatest extent.

### b. ARE TREATMENT DIFFERENCES CONSISTENT FROM TIME TO TIME?

This can be estimated by calculating the variance ratio for the mean square of treatments x times divided by that for treatments x blocks in times. These results are presented in Table 16.

Table 16. Ability of Grading Systems to Measure Treatment Differences Consistently

| Grading System | Variance Ratio |
|---|---|
| LG | 3.40* |
| MLG | 4.62** |
| 6VC | 2.98* |
| 6VO | 1.67 |
| 3VC | 1.89 |
| 3VO | 2.14 |

* Variance ratio is significant (P = 0.05).
** Variance ratio is highly significant (P = 0.01).

Since, for consistent performance, treatment differences in time must not differ significantly, one must conclude from Table 16 that treatment differences fluctuate significantly from time to time when leaf grading systems are used, but that variation is not significant when grades based upon discoloration in three vascular bundles are employed.

### c. WHAT IS THE EFFECT OF TIME UPON DISEASE AVERAGED OVER ALL TREATMENTS?

This can be measured through the variance ratio: mean square for times divided by that for treatments x times. As in the previous case, this variance ratio will be at a minimum for the ideal grading system. Results for the several systems are reported in Table 17.

Table 17. Effect of Time on Disease Averaged Over All Treatments

| Grading System | Variance Ratio |
|---|---|
| LG | 3.27[1] |
| MLG | 3.20[1] |
| 6VC | 1.31[1] |
| 6VO | 1.07[1] |
| 3VC | 1.00[1] |
| 3VO | 1.09[1] |

[1] Variance ratio is not significant.

Although the variance ratio is not significant for any grading system, the evidence suggests that the effect of time on disease is minimized when vascular grades are based upon three bundles in the stem.

### 4. Conclusions on Evaluation of Grading Systems

The immediately foregoing discussion has concerned itself with a comparison of eight grading systems which have been used in experiments on chemotherapy. Some of these comparisons have been made at one time; others are based on consistency of performance in time over winter and summer seasons.

By far the single most useful grading system is that designated in the foregoing discussion as the 3VO grade, and described in detail under procedure for reading vascular discoloration. Evidence has been presented which shows that variance ratios for treatments when 3VO grades are used exceed those for leaf grades in some cases, and are only slightly less than those for leaf grades in others. The 3VO grade is not greatly influenced by the time of year when it is used nor by positional effects of plants in the greenhouse bench; evidence is presented which shows that disease grades based on wilting of leaves are significantly affected by these causes.

Perhaps the second most useful grade of disease severity is the leaf grade itself. Although it has the shortcomings outlined above, this grading system evaluates treatments efficiently because treatment differences as well as the error term are greater than for the 3VO grade. Knowledge of the sensitivity of the leaf grade to positional and seasonal influences may be used to advantage in reducing variation in experiments.

## B. INTERRELATIONS OF THE GRADES

In the foregoing section, several grading systems were evaluated with the idea of arriving at a single grading system which would give the maximum information concerning the severity of disease for Fusarium wilt of tomato. These grading systems have also been evaluated in terms of their ability to discriminate between effective and ineffective chemotherapeutic treatments.

There remains the possibility that it may be useful to employ two grading systems simultaneously to obtain information which a single grading system would not give. For example, if the leaf grade were a

measure of the activity of lycomarasmin formed by *Fusarium* in the plant and if the vascular discoloration in the stem were a measure of the extent to which the fungus had actually invaded the plant, then the two grades would give qualitatively different kinds of information about the disease picture, and one might, by plotting one grade against the other, be able to infer modes of chemotherapeutic action from this relation. Thus, if a compound acted by inactivating lycomarasmin in the plant, the leaf grade would be lower than expected in terms of the amount of vascular discoloration in the plant, and a plot of the leaf versus the stem grades for a number of compounds would quickly identify compounds that were behaving in this way. It was with this possibility in mind that the interrelations of the various grades have been carefully studied.

### 1. Analysis of the Discriminant Function Among the Five Grading Systems: LG, PC, PO, 6VC, and 6VO[1]

The discriminant function analysis is designed to give evidence on precisely the point under discussion. Such an analysis will indicate which of a number of ways of measuring severity of Fusarium wilt, can be combined to provide additional information about either the disease picture or treatment effects which is not available from an analysis of one of the grades alone.

For this analysis an experiment was set up in which four compounds, all of them effective chemotherapeutants, were applied in either three or four dosages each to five plant replicates in a randomized block design. At harvest, plants were graded by each of the five grading systems: LG, PC, PO, 6VC and 6VO. Analysis for the discriminant function, details of which will not be given here, was made in the usual way. The interested reader is referred to Fisher (17) for a discussion of the objectives and procedures of the analysis.

This analysis selected the two grading systems PO and 6VC as the pair providing the greatest amount of additional information about the disease picture. This conclusion was startling at first, because it indicated that the leaf grades are closely related in character to the stem grades (see page 32), since leaf grades were eliminated early in the analysis. Nonetheless, the hypothesis was set up that the PO and 6VC grades are different in character and an effort was made to determine what kind of information could be deduced from a study of the interrelation of these grades. How they are related was learned from a comparison of the grades themselves, discussed on page 45.

### 2. Analysis of Correlation Coefficients Among the Grading Systems

The interrelation of the several grading systems was analyzed and, where grading systems were directly proportional to one another, correlation coefficients were calculated. In these analyses the MLG grading system was disregarded, since it is related to other kinds of grading systers in the same manner that LG is.

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[1] For a summary description of these grading systems, see Table 10, page 32.

For this analysis the same data were used as were employed in the study reported in Table 11. The experiment itself, described on page 14, consisted of an evaluation of 21 chemotherapeutic treatments, each applied to five replicate plants. When plants were graded for disease severity, they were rated in terms of each of the grading systems described in Table 10. Thus, there were 105 observations for each grading system and 21 treatment means for each.

### a. THE RELATION OF VASCULAR DISCOLORATION IN THE STEM AS MEASURED BY GRADES 6VC AND 3VC TO VASCULAR DISCOLORATION IN THE PETIOLE AS MEASURED BY GRADE PC

A comparison of treatment means for the 6VC and PC grades indicated that, when the plant was only slightly diseased, the petiole grade was slightly less than the stem grade was. When a plant was more than slightly diseased, however, this situation was reversed. These relations are shown in Figure 7 in which is plotted the difference between values of PC and 6VC against the corresponding values of 6VC.

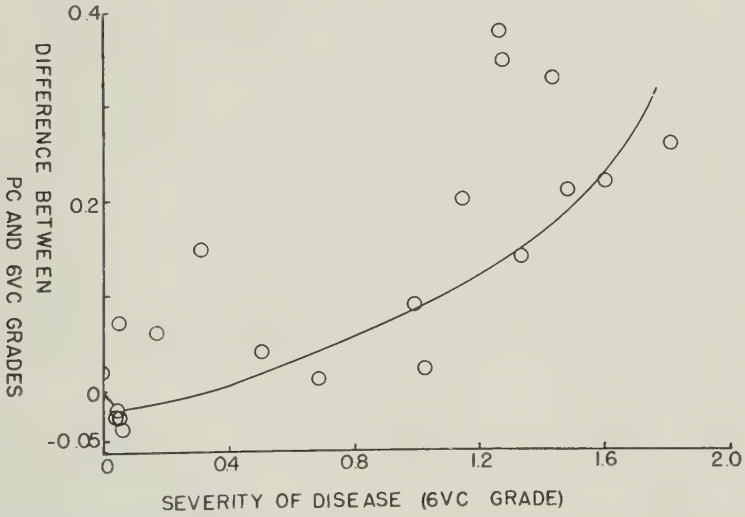


**Figure 7. The relation between PC - 6VC and the 6VC grade. For a summary of how these grades are taken, see Table 10.**

Since discoloration progresses up the stem and since the petiole is attached higher on the plant than bundles from which its traces are derived, one would expect the discoloration in petioles to be less than in the stem when discoloration in the stem is only slight. This effect is the result of decreasing amounts of discoloration from *Fusarium* with height of the plant.

Why discoloration in the petiole should be graded higher when discoloration in the stem is severe is not so obvious, but it can be accounted

for in terms of the nature of the two grading systems and the vascular anatomy of the tomato. One must bear in mind that the large bundles in the stem are easy to see and are quite readily graded for the presence and intensity of discoloration. In the case of the three small vascular bundles which alternate in the stem with the three large ones, the operator finds it quite difficult to grade for intensity of discoloration accurately. Sometimes he underestimates the score for color in small bundles relative to the score he assigns large ones, because the spot of color is smaller. When the spot of color is conspicuous, the operator gives the small bundles a high score. Hence, the average is based on more variable data when discoloration in small bundles is ignored. That this happens may be inferred from the analysis of variance for the 6VC grade in relation to the 3VC grade through a comparison of the F values for treatments and blocks, presented in Table 11.

The two pairs of lateral vascular bundles that traverse a petiole arise from large bundles in the stem (2, 38), whereas the median bundle is a continuation of a small bundle in the stem. One of the two lateral pairs of vascular traces in the petiole is so small as to be hardly visible to the naked eye and it has customarily been ignored in taking petiole grades. Thus, of the three bundles graded, two of them arise from large bundles in the stem. In contrast, for the 6VC grade three of the six bundles are large ones. Consequently, 2/3 of the petiole grade, PC, is based upon the discoloration in large bundles, while only 1/2 of the 6VC grade is derived from the behavior of large bundles. Because small bundles are more likely to be underscored than large ones and because the petiole grade is determined to a greater extent by the behavior of the large bundles than the stem grade is, the petiole grade will usually be higher than the stem grade when discoloration is moderate to high.

Knowing this, one would predict that when vascular grades in the stem are based on the intensity of discoloration in large bundles only (3VC grade), they should always receive a higher score than the petiole grade (PC). Examination of data in detail has shown that this is true except in rare cases.

Thus, the differences between the 6VC and PC grades are entirely resolvable in terms of trace anatomy and the errors in assigning grades based on intensity of discoloration in the small vascular bundles of the stem and petiole. The discrepancies between these two grades arise from errors in scoring small bundles for intensity of discoloration. Otherwise these grades measure the same factors. Apparently nothing can be learned about the mode of action of a chemotherapeutant from studying the interaction of the grades 6VC and PC. Since the 3VC grade is proportional to but always greater than the PC grade, it, too, is a direct measure of PC and little can be learned from its interaction with the PC grade.

### b. THE RELATION OF VASCULAR DISCOLORATION IN THE PETIOLE (PO) TO THAT IN ALL SIX BUNDLES IN THE STEM (6VO) WHEN INTENSITY OF DISCOLORATION IS IGNORED

The relation of the PO grade to the 6VO grade is one of direct proportionality (inset of Figure 8), and one would predict that this would

be so. The operator does not make a serious underscoring of small bundles by either system because he is not expected in this system to do more than state whether or not bundles are colored. Since the bundles in the stem are larger than in the petioles, discoloration is more readily seen there and the 6VO grade is usually slightly higher than the PO grade for a given treatment.

### c. THE RELATION BETWEEN VASCULAR DISCOLORATION IN THE PETIOLE BUNDLES, IGNORING INTENSITY OF COLOR (PO) AND THAT IN THE STEM, GRADING ALL SIX BUNDLES FOR INTENSITY OF DISCOLORATION (6VC)

Analysis of the discriminant function indicated that the grading systems PO and 6VC were of greater interest than any others in providing additional information about the pattern of disease symptoms in the plant or about modes of chemotherapeutic action. Before this pair of grades can be used with confidence to deduce modes of chemotherapeutic action, one must be certain that their high interaction is not caused by some other factor.

By comparing the 6VC grade with PO, one finds that the petiole grade tends to be less than the 6VC grade when discoloration is low and to be higher when discoloration is moderate to high. Thus, the relation between these two grades is the same as the relation between 6VC and PC grades (Figure 7). To illustrate this effect, it has been necessary to divide 6VC grades by three to correct the range of this grade to the range of the PO system. In Figure 8 the difference between the PO and the

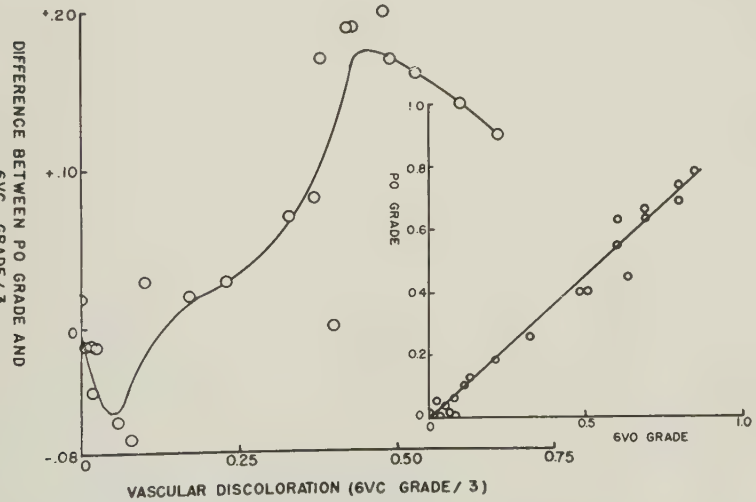


**Figure 8. The relation between PO - 6VC/3 and 6VC/3. For an explanation of how these grades are taken, see Table 10.**

**Inset: The relation between grades based on the number of vascular bundles showing discoloration in the petiole, PO, and those based on the number of vascular bundles discolored in the stem, 6VO.**

6VC grade divided by three is plotted against values of the 6VC grade divided by three. Here it can be seen that the relation of the two grading systems is similar to that shown in Figure 7, a complex relation ascribed in that case to difficulties in scoring for color in small bundles and the nature of the trace anatomy of the plant.

The discriminant function analysis selected these two grading systems as ones which have an interesting interaction. Evidently it is the anatomy of the plant and the nature of the scoring systems which bring about the "interesting" interaction. But this has nothing to do with modes of chemotherapeutic action and we may disregard this pair of grades as one that is useless in that connection.

### d. THE RELATION OF ALL OTHER GRADES BASED ON VASCULAR DISCOLORATION TO GRADE 3VO[1]

We have already seen that the measures of stem discoloration, grades 6VC and 3VC, are direct measures of the petiole grade PC, insofar as treatment effects are concerned. The grades 6VO and 3VO are directly related to PO in the same manner. Thus, discoloration in the stem is always a direct measure of discoloration in the petiole, excepting for scoring errors. We may conclude that any grade of vascular discoloration measures the same attributes of disease severity as any other vascular grade does. However, certain grades are more accurate than others because of their relative freedom from estimation errors. The 3VO grade is the best such measure.

One may obtain evidence that this is so by calculating the correlation coefficients which relate these grades, one to the other. This has been done for all vascular discoloration grading systems in comparison with the 3VO grade, as shown in Table 18.

Table 18. Correlation Coefficients of all Grading Systems in Relation to 3VO Grades

| Grading system[1] | Correlation with 3VO system | |
|---|---|---|
| | r | $r^2$ |
| PC | 0.973 | 0.95 |
| PO | 0.981 | 0.97 |
| 6VC | 0.971 | 0.95 |
| 6VO | 0.992 | 0.99 |
| 3VC | 0.985 | 0.97 |
| 3VO | 1.000 | 1.00 |

Table 18 shows a very high correlation between all other vascular discoloration grades and the 3VO grading system. The extent to which one grading system will give information available from the 3VO system is given by $r^2$ in Table 18. These values will be recognized as exceptionally high in experimental work. Hence, one grade to a high degree provides information concerning any other grade.

[1] See Table 10 for a summary description of these grading systems.

### e. THE RELATION OF THE VASCULAR DISCOLORATION IN THE STEM, GRADE 3VO, TO THE LEAF GRADE, LG

Finally there remains to be analyzed the extent to which grades 3VO and LG are related. In the experiment under discussion, the correlation coefficient between treatment means for 3VO grades and LG grades was calculated to be 0.958, a rather high degree of correlation. Taking the square of this value, *i. e.*, $r^2 = 0.92$, as a measure of the information available when one grade is used as a measure of the other, we see that little information is lost if leaf grades are used instead of 3VO grades or vice versa. The correlation coefficient between LG and 3VO, based on all 105 plots and not just upon treatment means, is 0.84.

In Figure 9 are plotted the treatment means for LG grades against the corresponding values of 3VO, from which the high degree of correlation is readily apparent. There are but two pairs of observations which do not fit the relation. An examination of the original data shows that these correspond to treatments C and E. Treatment E, it will be remembered, had two missing plots and values for them had to be assigned by the missing plot technique. The two plots were missing because the treatment was highly phytotoxic and two of five plants were killed. It will be noted that the leaf grade for this point is higher than would have been expected on the basis of the 3VO grade, considering the high correlation between 3VO and leaf grades. We may say with some assurance, therefore, that this point is artificially high because leaf grades measured wilting and phytotoxic symptoms together. Similarly, treatment C was noted as producing pronounced phytotoxicity during the treatment period, but none of the plants receiving this treatment was killed. If data on these treatments are eliminated in calculating the correlation coefficient, the resulting value is 0.990.

The leaf grade will evidently be subject to some treatment variation not ascribable to chemotherapeutic effects when compounds are used at phytotoxic concentrations. Therefore, it is important that compounds be applied at nonphytotoxic levels if leaf grades are to be used as a criterion of chemotherapeutic activity.

Obviously, then, leaf and 3VO grades are measures to a high degree of the same thing in the absence of foliar phytotoxicity and it appears that little can be learned from studying the interactions between these two grades for various treatments under these conditions. However, when miscellaneous compounds are under test, plots of the LC and 3VO grades for treatment means against one another may be useful in identifying phytotoxic treatments. When the leaf grade is much higher than expected as judged by the 3VO grade, the compound has probably been phytotoxic. Thus, the two points C and E in Figure 9 represent treatments known to have been phytotoxic. The operator clearly confused Fusarium wilt and phytotoxic symptoms in assigning grades to leaves in these cases.

There will be instances where it is helpful to obtain quantitative data on whether there are discrepancies in the information provided by the leaf and 3VO grades. An analysis of variance in which both grades are employed simultaneously will give this information. Such an analy-

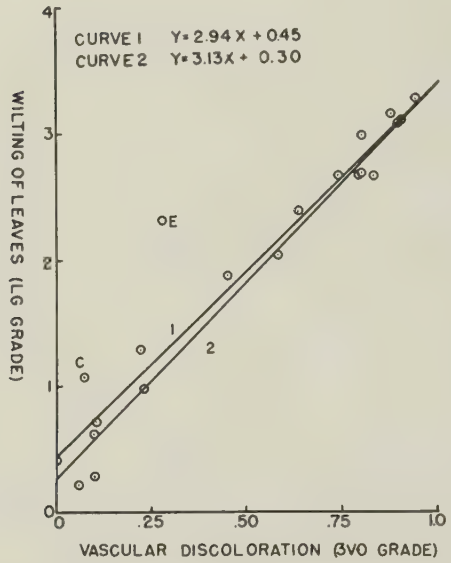


**Figure 9. The relation between severity of Fusarium wilt, measured by leaf grades, LG, and measured by the number of large stem bundles which are discolored, 3VO. Curve 1 is based on all 21 treatments, whereas curve 2 is fitted by ignoring the two phytotoxic treatments, C and E.**

sis has been made, using the grades obtained in the experiment analyzed in Table 11. In the present analysis it was convenient to divide leaf grades by four in order that the range of LG should become the same as for 3VO. The five 3VO and LG/4 grades were then used in a single analysis as independent observations in the manner and with the results shown in Table 19, using the standard method of analysis for split plots.

Table 19. Analysis of Variance of Disease Grades, Involving Both LG/4 and 3VO Estimates of Disease Severity

| Source of Variance | Degrees of Freedom | Mean Square | Variance Ratio |
|---|---|---|---|
| Total | 209 | | |
| Treatments | 20 | 0.9184[1] | 19.50** |
| Blocks | 4 | 0.3408[1] | 7.23** |
| Treatments x Blocks | 80 | 0.0471[2] | 8.90** |
| Grading Methods | 1 | 0.0116[3] | n. s. |
| Grades x Blocks | 4 | 0.0207[2] | 3.90** |
| Treatments x Grades | 20 | 0.0362[2] | 6.83 |
| Treatments x Grades x Blocks | 80 | 0.0053 | |

** Variance ratio highly significant (P = 0.01).
n.s. Variance ratio not significant.
[1] Treatments x Blocks term used as estimate of error in computing variance ratio.
[2] Treatments x Blocks x Grading methods term used as estimate of error in computing variance ratio.
[3] Grading methods x Blocks term used as estimate of error in computing variance ratio.

If leaf and 3VO grades are essentially measures of one and the same thing, the interaction of treatments x grading methods should not be significant, yet Table 19 indicates that it was. Examination of the sums of disease grades for the two grading systems showed that only two treatments, C and E, were responsible for the significance of this term. Evidence has already been presented that these were phytotoxic treatments and that foliar phytotoxicity was confused with wilt sumptoms in assigning leaf grades. In the case of the other 19 treatments, the two estimates of disease severity were in essential agreement. Thus, there is no evidence as yet to indicate that 3VO and LG are not identical measures of disease severity.

Such an analysis is valuable in finding whether the thesis presented here is correct and in finding whether (a) treatments have been applied at injurious concentrations or (b) there is evidence of a significant interaction between leaf and 3VO grades in the absence of phytotoxicity, an interaction of value in inferring the modes of chemotherapeutic action.

It has been suggested that the combination of LG and 3VO grades be used for another reason. Under field conditions, tomatoes will sometimes wilt heavily as a result of attack by *Fusarium* without showing heavy discoloration. In such cases the leaf grade will be safer to use than the 3VO grade. Again, some compounds, notably nitrites, may cause discoloration of vascular bundles in the absence of *Fusarium*. Both of these conditions would indicate that LG is an index that is safer than 3VO. We have already seen that 3VO, when such factors are not operative, is a more reliable and quantitative measure than LG. Will a margin of safety be introduced by using both of these grades?

A margin of safety may not be truly introduced when leaf and 3VO grades are used together to evaluate treatment effects. In the first place, a number of factors other than *Fusarium* may cause leaves to wilt or drop from the plant. Water shortage, temperature effects, transplanting shock, and phytotoxic components of the inoculum are all responsible for wilting and loss of leaves. In the second place, it is uncertain whether leaf grades are independent of 3VO grades when discoloration of vascular bundles is caused by factors other than *Fusarium*. Thus, discoloration produced by exposing a plant to nitrite may also cause wilting of leaves as a result of vascular dysfunction. Certain compounds have been noted which, on occasion, will cause vascular discoloration. These compounds also cause an increase in leaf grade which is proportional to the extent of vascular discoloration. Thus, the leaf grade may be affected by environment but in the absence of such effects the leaf grade will always be proportional to the vascular grade. Since very few factors will cause vascular discoloration without causing leaves to wilt or be dropped, the vascular grade will generally be a better measure of Fusarium wilt than any combination of the leaf and vascular grades. When compounds are used which cause vascular discoloration in their own right, they probably cannot be useful as chemotherapeutants, but the 3VO grade can be corrected by treating noninoculated plants and deducting the resulting score from the 3VO grade assigned inoculated plants.

### 3. Summary of the Interrelations of the Grades

We may conclude from the foregoing discussion that all of the grading systems are directly related to one another, insofar as treatment effects are concerned. Interactions between grades are apparently a result of the vascular trace anatomy of the tomato plant and of more variable scoring of small vascular bundles, whether they are scored for presence or for intensity of discoloration. Little seems to be gained by taking more than one grade and nothing can be learned (so far as present evidence shows) from a study of the relation of one grade to another, insofar as mode of chemotherapeutic action is concerned.

The 3VO grade has been shown to give a maximum variance ratio for treatments and to be least affected by position of the plant in the greenhouse bench and by seasonal effects.

The leaf grade, on the other hand, is subject to variation caused by seasonal factors or by positional effects and gives a somewhat smaller variance ratio for treatments. It is more likely than the vascular grade to give aberrant results when a phytotoxic compound is used.

## V. The Usefulness of Results of Chemotherapy Assays

There are at least three ways in which data on the performance of compounds in the Fusarium wilt assay on tomatoes is useful in further research on chemotherapy. With a quantitative assay procedure, it is possible to study the relation of chemical structure to chemotherapeutic activity, once a promising structure is found. Having found a number of different chemical structures possessing chemotherapeutic activity, one can examine their probable modes of action. Finally, one can use the results of assays to predict the performance of compounds either on the disease on which the assay was conducted or on other diseases when these compounds are used under field conditions.

### A. THE RELATION OF CHEMICAL STRUCTURE TO CHEMOTHERAPEUTIC ACTIVITY

It is not the purpose of the present paper to enter into a detailed discussion of the chemistry of promising chemotherapeutants, but it may prove useful to explore the extent to which the assay method may be employed in evaluating the effect of substituents upon the activity of a central nucleus showing promise. For this purpose benzene sulfonanilide and some of its derivatives will serve as an illustration. These compounds were evaluated in a series of assays. To render the results comparable from one assay to another, results are expressed in terms of the chemotherapeutic ratio. This is defined as the ratio of the mean 3VO grade for a given treatment to the corresponding grade for check plots in the same assay and it corrects for variations occurring from uncontrolled sources from one assay to another. The relation of structure of a series of benzene sulfonanilides[1] to their phytotoxicity and chemotherapeutic potency is given in Table 20.

[1] Supplied by Rohm and Haas Co., Washington Square, Philadelphia, Pa.

Each compound was assayed at the maximum concentration that was not seriously phytotoxic. The concentration of a compound that a plant will tolerate is therefore an inverse measure of inherent phytotoxicity of the compound. Thus, when plants tolerate a concentration of 1000 ppm of a given compound, it is inherently less phytotoxic than a compound which must be applied at 8 ppm to avoid injury. Similarly, the chemotherapeutic ratio is an inverse measure of chemotherapeutic potency. When the ratio was zero, there was no discoloration in stems of treated plants and when the ratio was 1.0, the disease in treated plants was the same as in the checks. With this in mind, one may evaluate the effects of substitution in various ways upon the chemotherapeutic potency and phytotoxicity of a series of benzene sulfonanilides.

### 1. The Effect of Substitution on Ring 1

Among the compounds for which ring 2 is unsubstituted, the following relations may be noted:

a. Nitro substitution on ring 1 markedly increases phytotoxicity while decreasing chemotherapeutic potency (HD 116 vs. HD 131).

b. 4-Bromo substitution decreases chemotherapeutic potency without affecting phytotoxicity (HD 227 vs. HD 114).

c. The increase in molecular weight brought about by substituting on ring 1 with inert groupings does not influence either chemotherapeutic or phytotoxic potency. Also, position of substitution is unimportant. This applies to substitution by methyl, chloro or acetamide groupings (HD 227 vs. HD 122, HD 121, HD 116 and HD 136).

d. Whether ring 2 contained substitutions on it or not, substitution of phenoxy or *p*-bromophenoxy groupings in position 4 on ring 1 destroys chemotherapeutic potency completely, but phytotoxicity remains essentially unaltered (HD 227 vs. HD 110, HD 111, HD 112 and HD 113).

### 2. The Effect of Substitution on the Sulfonanilide N

Benzene sulfonanilide behaves as an acid and will form salts. When reactions depending upon its acidic properties are involved, the following relations may be noted:

a. The soluble sodium salt possesses increased phytotoxicity but chemotherapeutic potency is decreased relative to the free acid (HD 125 vs. HD 124).

b. Insoluble salts are less phytotoxic but have more chemotherapeutic potency than the free acid (HD 25 vs. HD 129).

c. The amide is less phytotoxic than the free acid but about equally potent as a chemotherapeutant.

These relations suggest that the active hydrogen on the sulfonanilide nitrogen is associated with phytotoxicity but not primarily with chemotherapeutic activity.

TABLE 20. THE CHEMOTHERAPEUTIC POTENCY AND PHYTOTOXICITY OF SOME BENZENE SULFONANILIDES[1]

| | Structure[2] | | | | Concentration[3] (ppm) | Chemotherapeutic[4] ratio |
|---|---|---|---|---|---|---|
| | Substituents on ring No. 1 | | Substituents on ring No 2 | | | |
| HD 227 | | $\phi$-$SO_2$-NH-$\phi$ | | | 500 | 0.12 |
| HD 114 | 4-Br- | " | | | 500 | 0.49 |
| HD 115 | 4-Br- | " | -Cl-4 | | 250 | 0.37 |
| HD 121 | 4-Me- | " | | | 1000 | 0.10 |
| HD 110 | 4-$\phi$-O- | " | | | 500 | 1.00 |
| HD 111 | 4-$\phi$-O- | " | -Cl-4 | | 500 | 0.99 |
| HD 112 | 4′-Br-4-$\phi$-O-(Br)X- | " | | | 1000 | 0.64 |
| HD 113 | 4′-Br-4-$\phi$-O-(Br)X- | " | -Cl-4 | | 1000 | 0.74 |
| HD 122 | 4-Me-CO-NH- | " | | | 1000 | 0.00 |
| HD 123 | 4-Me-CO-NH- | " | -Cl-2 | | 500 | 0.98 |
| HD 136 | 2,5-$Cl_2$- | " | | | 1000 | 0.18 |
| HD 135 | 2,5-$Cl_2$- | " | -$NO_2$-3 | | 125 | 0.46 |
| HD 134 | 2,5-$Cl_2$- | " | -$NO_2$-4 | | 32 | 0.43 |
| HD 25 | 3,4-$Cl_2$ | " | -$NO_2$-4 | | 32 | 0.13 |
| HD 116 | 3,4-$Cl_2$- | " | | | 500 | 0.16 |
| HD 117 | 3,4-$Cl_2$- | " | -Cl-4 | | 63 | 1.01 |
| HD 133 | 3,4-$Cl_2$- | " | -$NO_2$-3 | | 63 | 0.45 |
| HD 130 | 3,4-$Cl_2$- | " | -$NO_2$-2 | | 1000 | 0.55 |
| HD 131 | 3,4-$Cl_2$-X-$NO_2$- | " | | | 16 | 0.49 |
| HD 118 | 3,4-$Cl_2$- | " | -Me-4-$NO_2$-3 | | 1000 | 0.89 |
| HD 119 | 3,4-$Cl_2$- | " | -Me-6-$NO_2$-3 | | 250 | 0.85 |
| HD 120 | 3,4-$Cl_2$- | " | -$Me_2$-4,6-$NO_2$-3 | | 500 | 1.10 |
| HD 124 | di iso propyl- | " | -$NO_2$-4 | | 1000 | 0.00 |
| HD 125 | di iso propyl- | $\phi$-$SO_3$-N(Na)$\phi$ | -$NO_2$-4 | | 63 | 0.74 |
| HD 97 | 4-Cl- | " | -$NO_2$-4 | | 8 | 0.62 |
| HD 129 | 3,4-$Cl_2$- | $\phi$-$SO_2$-N(Cu/$_2$)-$\phi$ | -$NO_2$-4 | | 63 | 0.00 |
| HD 128 | 3,4-$Cl_2$- | $\phi$-$SO_2$-N($COCH_3$)-$\phi$ | -$NO_2$-4 | | 1000 | 0.24 |

NOTE: Footnotes for this table appear on opposite page.

### 3. The Effect of Substitution on Ring 2

Substitution on ring 2 produces a number of interesting effects on chemotherapeutic potency as contrasted with phytotoxicity. Among these are the following:

a. The addition of a nitro group in the 4 position on ring 2 increases phytotoxicity (HD 97 vs. HD 114).

b. Chlorine substitution in the 2 position on ring 2 destroys chemotherapeutic activity (HD 227 and HD 122 vs. HD 123). Substitution in the 2 position by either Cl or $NO_2$ destroys chemotherapeutic activity and reduces phytotoxicity (HD 130 vs. HD 116, and HD 227 and HD 122 vs. HD 123).

c. Compounds having 4-chloro substitution in ring 2 have lower phytotoxicity and chemotherapeutic potency than similar compounds which have 4-nitro substitution (HD 117 vs. HD 25). However, compounds having 4-nitro substitution on ring 2 appear to be generally more phytotoxic than those with an unsubstituted ring 2.

d. For a series of ring 1 3,4-dichloro derivatives, the order of decreasing chemotherapeutic potency among compounds with substitution on ring 2 is 4-$NO_2$ (HD 25), none (HD 116), 3-$NO_2$ (HD 133),2-$NO_2$ (HD 130), and 4-Cl (HD 117). In contrast, the order of decreasing phytotoxicity among these compounds is 4-$NO_2$, 4-Cl, none, 3-$NO_2$ = 2-$NO_2$. These differences between phytotoxicity and chemotherapeutic potency suggest that the active centers for phytotoxicity are different from those for chemotherapeutic activity. Other evidence for this is the wiping out of chemotherapeutic activity by 4-Cl substitution in ring 2 while $NO_2$ substitution at this position does not affect it, although phytotoxicity is unchanged (HD 25 vs. HD 117).

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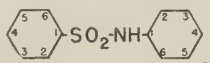
[1] Many of these sulfonanilides were synthesized by the Rohm and Haas Co. especially for this study. All of them were obtained from Rohm and Haas Co., Washington Square, Philadelphia, Pa.

[2] $\phi$ stands for phenyl. Structure of the central nucleus is:




Position of substituents is indicated by prefixes and suffixes. The ring on which substitution occurs is indicated by whether substituents are indicated by a prefix (ring 1 substitution) or a suffix (ring 2 substitution). The following abbreviations are used: Me for methyl, Cl$_2$ for dichloro, Me$_2$ for dimethyl, NO$_2$ for nitro, $\phi$-O for phenoxy, and X denotes position of substitution is unspecified.

[3] Compounds assayed at maximum nonphytotoxic concentration.

[4] Chemotherapeutic ratio is computed from 3VO grade for treated plots by dividing by 3VO grade for check plots.

### 4. The More Substitution on Ring 2, the Lower Is Phytotoxicity and Chemotherapeutic Potency

The foregoing discussion serves to indicate how the results of assay may be useful in noting the relations of chemical structure to activity. From these results predictions may sometimes be made. An example is the prediction of chemotherapeutic activity for benzene sulfonanilide, HD 227. Compounds in the series presented in Table 20 were assayed in serial order, by the HD code number. On the basis of these investigations, HD 227 was predicted to be as potent as any compound in the series and to be lower in phytotoxicity than many. It was therefore obtained and its potency assessed. The prediction of its biological properties was borne out, as may be seen in Table 20.

## B. THE MODES OF CHEMOTHERAPEUTIC ACTION

A second objective of assessing the chemotherapeutic value of compounds involves learning more about the mechanisms by which they reduce the severity of disease in the plant. As was stated earlier, certain mechanisms have been conceived as likely while others are not yet fully appreciated as potential mechanisms. By assembling a number of compounds which act as chemotherapeutants and by then studying how they produce the effects that they do, it will be possible to determine what mechanisms are involved in chemotherapy, how many mechanisms occur among compounds, which mechanisms are of most frequent occurrence, and which offer promise of reducing disease severity to the greatest extent. The results of studies of this nature will be applicable to other diseases and need not be restricted in their application to Fusarium wilt. Some of these mechanisms will be discussed in Section VII.

## C. PREDICTING THE FIELD PERFORMANCE OF CHEMOTHERAPEUTANTS

Fusarium wilt of tomatoes was deliberately chosen as the basis for this chemotherapeutic assay. Information was desired on the chemotherapy of vascular wilt diseases, particularly Dutch elm disease. Of the many hosts for such diseases, tomatoes are as readily grown as any plant and far more simply than most. A primary assay based upon elms would be prohibitively extravagant of time and space. Fusarium wilt was chosen because, as expressed in the tomato plant, it lends itself to a quantitative grading, thus making it possible to discriminate differences nicely. Verticillium wilt, on the other hand, frequently produces a generalized discoloration in plants rather than a discoloration that is restricted to primary vascular bundles; this pattern is quite impossible to grade in a quantitative way in any manner comparable to what can be done with Fusarium wilt on tomatoes. Unfortunately, it is not possible under Connecticut conditions to test the predictability of performance of compounds on Fusarium wilt of tomatoes under field conditions, since summer soil temperatures are usually not sufficiently high that the disease is a field problem. Even inoculated plants frequently fail to succumb to the disease because of this fact. For this reason, it has been necessary

to explore the performance of chemotherapeutants selected by the *Fusarium* assay on other hosts and with different diseases.

## 1. General Properties of Chemotherapeutants Which Affect Their Activity

The performance of compounds on other diseases can to some extent be predicted from information acquired on them in terms of Fusarium wilt, especially as it pertains to such properties as stability in soil and how the compound affects the plant.

### a. BREAKDOWN OF COMPOUNDS IN THE SOIL

In Section III-G data have been given which illustrate that some compounds are unstable when applied to soil and, though effective when applied to tomatoes growing in sand, are quite ineffective when applied to tomatoes growing in soil. In Figure 5 the performance of two compounds was reported: CC 1182 and HD 25. In this figure it was shown that CC 1182 was active under both conditions, whereas HD 25 was ineffective when applied to soil.

These same compounds have been applied to chrysanthemums for control of Verticillium wilt. They were applied to 10 plants each, five of which were grown in sand and five in soil. After the usual 10-day period of treatment, plants were inoculated by the toothpick method. After about six weeks, they were graded for severity of disease, and it was found that the performance of treatments was exactly as it had been on tomatoes: CC 1182 was effective on plants grown either in sand or soil, while HD 25 was ineffective in soil but was effective when applied to plants growing in sand. Evidently, the general behavior of two compounds on tomato wilt could be used as a basis for predicting performance on Verticillium wilt of chrysanthemums.

### b. COMPOUNDS CAUSING ROOT INJURY TO PLANTS

Compounds which injure the roots of tomatoes can reduce the severity of Fusarium wilt in chemotherapy assays. Keyworth and Dimond (34) have shown that when roots of tomato plants are injured, the metabolism of the plant is altered with resultant increase in reducing sugar levels and decrease in the amounts of potassium, calcium and phosphorus in the plant. These results suggest a mechanism for chemotherapeutic action to which little attention has been paid in the past. To what extent does the root-injuring capacity of compounds affect the susceptibility of other plants to disease?

In field experiments on Verticillium wilt of eggplant conducted in 1951, four of 15 compounds which caused root injury reduced the severity of wilt. Plants were placed in a soil infested with Verticillium and treatment was begun as soon as plants were placed in the field. Five applications were made once a week and five weeks after the last application, the severity of Verticillium wilt was assessed by grading each plant as a unit. The percentage of foliage which showed wilting symptoms was graded by the Horsfall-Barratt system (26, 27). Of the 15 treat-

ments, the four which caused root injury had suppressed Verticillium wilt to the greatest extent; moreover, the extent of disease suppression was in the same order as the severity of dwarfing of the plant from root injury. Quite evidently root injury on eggplant also reduces its susceptibility to Verticillium wilt. Information on the root-injuring capacity of compounds may be used as a basis for predicting their field performance when the plant is known to be made more resistant to disease by root injury. However, when root injury is great, treatments are unlikely to be useful in practice.

## 2. Performance of Specific Compounds on Vascular Wilt Diseases

Any assay of chemotherapeutic potency will be limited in usefulness unless the performance of compounds can be predicted with reasonable accuracy in terms of field conditions. But any prediction is less reliable as the departure from conditions of the assay becomes greater. The Fusarium wilt assay is no exception. If the Fusarium wilt assay is to be useful, it should give results which can be used as the basis for prediction of performance of Fusarium wilt under field conditions. Similarly, these results ought to be useful in predicting the performance of chemotherapeutants for other vascular wilt diseases, the pathogen of which, like *Fusarium*, is soil borne and enters the host through mechanically injured roots. Vascular wilt pathogens which enter the host above ground may not be so well controlled by compounds active against Fusarium wilt because the locus of infection is more remote from the treated roots when soil applications of chemotherapeutant are made.

To estimate the reliability of predictions, the relative performance of compounds in the Fusarium wilt assay has been compared with their performance when used as chemotherapeutants against other diseases. In seeking this correlation, one expects similar rather than identical performance. Compounds have been ranked in decreasing order of efficiency as chemotherapeutants, in the tomato wilt assay and in field experiments involving other diseases. Correlation coefficients have been computed between assay and field ranks, and the likelihood of a coefficient of correlation as great as this arising from chance has been estimated.

### a. PREDICTIONS OF THE PERFORMANCE OF CHEMOTHERAPEUTANTS ON SOIL BORNE VASCULAR WILT DISEASES

Chemotherapeutants promising in the tomato wilt assay have been used in field experiments for two soil borne vascular wilt pathogens: (a) Fusarium wilt of carnations and (b) Verticillium wilt of eggplant.

#### *(1) Fusarium Wilt of Carnations*

Fusarium wilt of carnations, caused by *Fusarium dianthi*, is closely parallel in its behavior to Fusarium wilt of tomato. Both diseases are soil borne. Both pathogens enter the plant through the roots, especially through wounded roots. Each affects the plant similarly. Accordingly, one might expect a reasonably good correlation in the effectiveness of

chemotherapeutants between the results of the tomato wilt assay and those obtained on Fusarium wilt of carnations.

In studying this correlation 10 compounds having satisfactory chemotherapeutic potency, as measured by the tomato wilt assay, were selected. Fifty carnation plants were grown in sand and 50 in soil and five plants of each lot were treated with each compound, according to the usual 10-day schedule. Plants were then uprooted, the roots washed, and the plants replanted, this time all in soil to which had been added an inoculum of *Fusarium dianthi*, grown on bran.[1] Six weeks after inoculation, plants were rated for the severity of disease, considering the plant as a whole, on a scale from 0 to 4. Results of treatment, together with the chemotherapeutic ratios, as measured by the tomato wilt assay, are given in Table 21.

TABLE 21. THE RELATION OF PERFORMANCE OF COMPOUNDS ON FUSARIUM WILT OF TOMATO (*F. oxysporum* f. *lycopersici*) TO RATINGS ON FUSARIUM WILT OF CARNATIONS (*F. dianthi*)

| Compound | Concentration (ppm) | Chemotherapeutic ratios | | |
|---|---|---|---|---|
| | | Tomato grown in sand | Carnations grown in sand | Carnations grown in soil |
| HD 3 | 500 | 0.04 | 0.85 | 0.50 |
| HD 95 | 64 | 0.23 | 0.70 | 0.40 |
| HD 109 | 250 | 0.00 | 0.35 | 0.45 |
| HD 121 | 1000 | 0.17 | 0.70 | .....[1] |
| CC 995 | 500 | 0.14 | 0.05 | 0.75 |
| CC 1041 | 250 | 0.26 | 1.35[2] | 1.00[2] |
| CC 1180 | 250 | 0.00 | 0.85 | 0.60 |
| CC 1182 | 33 | 0.00 | 0.00 | 0.75 |
| CC 1211 | 125 | 0.20 | 0.55 | 0.80 |
| CC 4016 | 500 | 0.22 | 1.00 | 1.00 |

[1] No plants in soil were treated with HD 121.
[2] Plants treated with CC 1041 showed no phytotoxicity when treatments were completed but were completely discolored, presumably from Fusarium wilt, two weeks after being inoculated.

Treatments were then ranked in decreasing order of effectiveness and numbers were assigned these ranks. The correlation coefficient was then computed between performance in the tomato assay and in the present experiment for carnations grown (1) in sand and (2) in soil. These coefficients of correlation are, respectively, 0.600 and 0.424. The former is significant at the 5 per cent level while the latter falls considerably short of significance, that is, in the former case the odds are no greater than 1:20 that an equally high correlation coecient could have arisen from chance, while in the latter case there are better than 1:20 odds that such a correlation could have arisen as a result of chance. Thus, reasonable predictions of performance of chemotherapeutic treatments can be made from tomato wilt data for chemotherapy of Fusarium wilt of car-

[1] This method has been found experimentally to be well suited for inoculating carnations with *Fusarium*, since it assures infection unless plants are well protected chemotherapeutically, and gives quite uniform disease response among a series of replicate plants.

nations when plants are grown in sand. These predictions are likely to be of little value when used on plants growing in soil, however, unless compounds inactivated by soil are first eliminated.

Stoddard and Dimond (60) have already reported the results of chemotherapeutic experiments on Fusarium wilt of carnations under commercial conditions. In this case compounds were applied to small plants twice a week early in the growing season and only once a week after plants were finally benched. 8-Quinolinol sulfate, CC 1182 (4-chloro-3,5-dimethyl phenoxy ethanol) and CC 1207 (2-norcamphane methanol), the three compounds used in the experiment, all controlled Fusarium wilt of carnations. These compounds had previously given moderate to high levels of control of Fusarium wilt as chemotherapeutants in the tomato assay.

In conclusion, then, there appears to be a significant correlation between the performance of compounds in the tomato wilt assay and their ability to act as chemotherapeutants on carnations for Fusarium wilt. The more comparable conditions are kept, the better predictions will be. When compounds are not first tested for their stability when applied to soil, predictions will be less reliable than when unstable compounds are eliminated before predictions are made.

### (2) *Verticillium Wilt of Eggplant*

The eggplant seems far more subject to phytotoxic injury than does the tomato and the concentrations of chemotherapeutants must be adjusted to allow for differences in susceptibility of the two species of plants. As has been noted, compounds which cause root injury produce a plant less susceptible to Verticillium wilt.

In experiments designed to measure the correlation between results of the tomato assay and the results of field experiments with chemotherapeutants for control of Verticillium wilt, a series of 15 compounds was used. These compounds were applied to the soil as soon as plants were set into the field, since the soil was naturally infested with *Verticillium*. Applications were made once a week for five weeks and three weeks after the last application plants were graded for severity of wilt. Thereafter, they were graded at intervals of approximately 10 days during the balance of the growing season. Grading was by the Horsfall-Barratt system (26, 27).

For readings taken on a given date treatments were ranked in order of effectiveness, numbers were assigned to these ranks, and the correlation coefficient computed between the ranking order of treatments in the Fusarium wilt assay and that obtained in the field on Verticillium wilt. The ranking orders of treatments in the tomato wilt assay and in the eggplant wilt experiment on three dates are given in Table 22, together with the coefficients of correlation on these dates. One would expect the effectiveness of treatments to diminish with time and the correlation to become poorer as the season advanced. As may be seen in Table 22, this is exactly what happened, and the highly significant correlation coefficients ($P=0.01$) obtained in early August decreased to values which

were significant only at the 5 per cent level by early September. However, the correlation in performance between the tomato wilt assay and the field experiment on eggplant wilt was still significant at the end of the growing season.

TABLE 22. THE PERFORMANCE OF CHEMOTHERAPEUTIC TREATMENTS IN THE TOMATO WILT ASSAY AND IN FIELD EXPERIMENTS ON VERTICILLIUM WILT OF EGGPLANT

| Compound | Ranking order of chemotherapeutic treatments | | | |
|---|---|---|---|---|
| | Tomato wilt | Verticillium wilt of eggplant on: | | |
| | | Aug. 10 | Aug. 16 | Sept. 8 |
| HD 3 | 4 | 7 | 8 | 6.5 |
| HD 25 | 5.5 | 15 | 12 | 15 |
| HD 57 | 13 | 13.5 | 10.5 | 1 |
| HD 80 | 2 | 3 | 4.5 | 2.5 |
| HD 95 | 8 | 2 | 1 | 12 |
| HD 100 | 11 | 11 | 13.5 | 13 |
| HD 109 | 1 | 1 | 2 | 4.5 |
| HD 115 | 12 | 11 | 9 | 4.5 |
| HD 116 | 7 | 7 | 4.5 | 10.5 |
| HD 121 | 5.5 | 7 | 3 | 8.5 |
| HD 126 | 10 | 9 | 10.5 | 2.5 |
| HD 136 | 9 | 4.5 | 7 | 10.5 |
| HD 139 | 3 | 4.5 | 6 | 6.5 |
| 8-Quinolinol benzoate | 14 | 11 | 15 | 8.5 |
| Check | 15 | 13.5 | 13.5 | 14 |
| Correlation coefficients | ($r_s$) | 0.688** | 0.705** | 0.550* |

** Correlation coefficient highly significant. ($P = 0.01$).
* Correlation coefficient significant ($P = 0.05$).

The rank correlation between performance of compounds in the tomato assay and as field chemotherapeutants on eggplant was found to be 0.688 for the August 10 reading, 0.705 for the August 16 reading and 0.550 for the reading taken on September 8. The first two values given are highly significant (odds of 1:100 that so high a correlation coefficient could arise by chance) and the value obtained from readings taken at the end of the season was significant (odds of 1:20).

We may conclude, therefore, that while predictions are not accurate in detail, those compounds that perform well on tomatoes will in general perform well on eggplant, and that use of the tomato assay is efficient for selecting compounds for field use on Verticillium wilt of eggplant.

### b. RELATION OF PERFORMANCE OF CHEMOTHERAPEUTANTS IN THE TOMATO ASSAY TO THAT ON DUTCH ELM DISEASE

Dutch elm disease differs from the other vascular wilt diseases discussed thus far. In the case of Verticillium and Fusarium wilts, the pathogen is soil borne and enters the host through its roots. When chemotherapeutic soil treatments are applied, the pathogen encounters roots that have been permeated by the chemotherapeutant. With Dutch elm disease, the fungus enters through wounds made in twigs by feeding of the elm bark beetle, and the point of entry is usually rather high aboveground.

The principal point of resemblance of Dutch elm disease to Fusarium wilt of tomato is that the pathogen probably acts upon the plant similarly to produce wilt. To be effective against Dutch elm disease, the chemotherapeutant must be translocated many feet to the infection court of the pathogen or its effects on host resistance must extend to the infection court. Data in Table 6 indicate that the effect of chemotherapeutants on resistance to disease may be local. For these reasons, it is possible that compounds which are effective against tomato wilt will be of little value against Dutch elm disease when they are applied to the soil.

Over a period of several years, compounds that have shown rather high chemotherapeutic potency for tomato wilt have been applied as soil treatments to elms for the control of Dutch elm disease. Of more than 50 such compounds, none has shown promise as a chemotherapeutant against Dutch elm disease when applied as a soil treatment.[1] Therefore, there is either no correlation or else other factors, not yet evaluated, must be weighed with the information provided by the tomato wilt assay to provide a useful basis for predicting performance of compounds as chemotherapeutants for Dutch elm disease.

In an attempt to resolve this difficulty, Chapman (3) compared the attributes of several chemotherapeutants for Fusarium wilt of tomato with those of 8-quinolinol benzoate, a chemotherapeutant for Dutch elm disease. The latter compound is absorbed by the plant and is translocated, as has been demonstrated by chemical analysis (11). Three compounds that were active against Fusarium wilt of tomato, CC 1182, HD 3 and HD 25, were compared with 8-quinolinol benzoate in terms of the zone of inhibition which might be formed about them on filter paper pads, using the method described by Thornberry (61). When either *Ceratostomella ulmi* or *Fusarium oxysporum* f. *lycopersici* were used as seeding material for the agar plates, no zone of inhibition was formed by the three compounds active against Fusarium wilt, but a definite zone of inhibition was formed by 8-quinolinol benzoate. To account for this, Chapman suggested that the three compounds that failed to form a zone of inhibition were bound on the cellulose fiber in the filter paper by adsorption. He suggested further that these compounds might be adsorbed on cellulose fibers in the plant or by other plant components that have comparable adsorbing capacities. Apparently the three compounds that are effective against Fusarium wilt of tomato are not translocated in the plant, whereas 8-quinolinol benzoate is.[2]

If this reasoning is correct, the Fusarium assay may favor chemotherapeutants that accumulate in roots and underestimate the absolute value of chemotherapeutants that are translocated to aboveground portions of the plant. Accordingly, the compounds favored by the tomato wilt assay may never reach the infection court in elms but will remain in the roots. Such compounds would be useless as chemotherapeutants against Dutch elm disease.

[1] 8-Quinolinol benzoate is no exception to this statement, since it is of little value against Fusarium wilt of tomato, although it is moderately effective as a chemotherapeutant against Dutch elm disease.

[2] CC 1182 causes a formative effect upon tomato foliage. Chapman (3) suggested that the quantity of material necessary for producing a formative effect was far less than the amount necessary to produce a chemotherapeutic effect and that the principal quantity of material remained bound in root tissue.

The problem, then, is one of determining (a) whether chemotherapeutants, as selected by the Fusarium wilt assay, fail when used in elms because the host and pathogen are qualitatively different in character or (b) whether the chemotherapeutants selected by the tomato assay fail in elms because they are not translocated to the infection court. Pertinent evidence on these points may be obtained by applying chemotherapeutants to foliage in such a manner that they will be absorbed into the plant. If a reasonable correlation then appears between results on tomatoes and elms, the previous failure of correlation resulted from nontranslocability of chemotherapeutants. On the other hand, if the correlation still is poor, one is inclined to consider that Fusarium wilt differs so much in character from Dutch elm disease that it cannot be used in light of present knowledge for predictions of chemotherapy for the latter disease.

Knight *et al* (36) have shown that oils in quick-breaking emulsions enter through stomates and that they may carry oil-soluble toxicants with them into the leaf. Accordingly, chemotherapeutants were dissolved in a nonphytotoxic oil and so formulated as to make a quick-breaking emulsion in water. During the summer of 1951, trees received foliage sprays at weekly intervals for five weeks with 13 compounds formulated in this manner. Plots were laid out as randomized blocks, with four trees per plot and five plots per treatment. After treatment, plants were inoculated with *Ceratostomella ulmi* and they were then graded for disease severity as the season progressed.

The value of chemotherapeutic treatment on elms was assessed in the same manner that results of eggplant treatments were evaluated.

Such an evaluation reveals no sign of significant correlation between the results of tomato wilt assay and field trials of compounds as chemotherapeutants for Dutch elm disease. Thus, the correlation coefficient between tomato assay of compounds and their value against Dutch elm disease was 0.154 on August 10 and was 0.21 on September 8.

The most promising chemotherapeutant yet encountered for Dutch elm disease was discovered in this experiment. This compound, the potassium salt of 2-carboxymethyl mercaptobenzothiazole (HD 109), was the only compound in the series under test which was soluble in water and insoluble in oil. Evidently, it penetrated in water solution into the leaves and/or the effects it produced extended as far as the infection court. It appears, then, that water soluble compounds of certain types penetrate leaves in aqueous solution better than oil soluble materials do in oil. Here, again, is evidence that the *Fusarium* assay has given preference to compounds which remain more or less localized in roots when treatment is applied to roots (see Table 6 and page 20).

Salts of 2-carboxymethyl mercaptobenzothiazole performed excellently as chemotherapeutants in the tomato wilt assay (Figure 2). The

**Figure 10. Appearance of elms treated with HD 109, the potassium salt of 2-carboxymethyl mercaptobenzothiazole, as a foliage spray for chemotherapy of Dutch elm disease.**

**Figure 11. Appearance of elms receiving no chemotherapeutic treatment for Dutch elm disease.**

effect in reducing Dutch elm disease is shown in Figures 10 and 11. The appearance of a plot treated with the potassium salt and then inoculated is shown in Figure 10. The appearance of an inoculated check plot is shown in Figure 11. Such differences appeared in all the five replicate plots.

### 3. Conclusions

When compounds are used chemotherapeutically under circumstances closely analogous to the conditions of the *Fusarium* assay, reasonably good predictions of their performance may be made. Data have been presented showing that good correlation exists between the results of the tomato wilt assay and those obtained on Fusarium wilt of carnations, on the one hand, and on Verticillium wilt of eggplant under field conditions, on the other. Both of these diseases represent ones in which the infection court is treated with the chemotherapeutant.

On the other hand, when compounds are used under conditions which are not comparable with those of the tomato assay, more information will be needed before valid predictions can be made. For example, compounds do not perform chemotherapeutically for Dutch elm disease in the same manner as they do for Fusarium wilt of tomato. In the tomato the infection court is permeated with chemotherapeutant; in the elm it is not. This may have bearing upon the poor correlation.

## VI. An Evaluation of Assay Methods for Assessing Chemotherapeutic Potency

The Fusarium wilt assay for chemotherapy has been presented in detail and its results have been shown to be useful in several types of study bearing upon chemotherapy. It will now be useful to make a comparative evaluation of the three principal assay methods which have been used in research on chemotherapy, indicating the conditions under which each assay yields most valid results. Primarily this discussion will concern itself with the assay method *per se* and will deal with modes of chemotherapeutic action only where assay methods have been developed in terms of a mode of action. Modes of action will be discussed in the section to follow (Sec. VII). We shall be concerned here with the assay based on neutralizing toxins as developed by Feldman, Caroselli and Howard (15), the assay developed by Crowdy and Wain (5,6) which uses *Botrytis fabae* on *Vicia faba,* and with the Fusarium wilt assay.

### A. NEUTRALIZATION OF TOXINS

Plant pathologists generally appreciate that toxic products of fungus activity may be a factor in pathogenesis, and the work of Howard and his coworkers (15, 31) has focused our attention on the possibility of alleviating this condition by specifically neutralizing such compounds with chemotherapeutants. This approach to disease control has stimulated the imagination of research workers and, when used properly, can

be a powerful approach to chemotherapy. Moreover, it can be made a rapid, quantitative assay which gives information on one mode of chemotherapeutic action while evaluating compounds.

Toxic products of a pathogen have been shown to cause wilting in the host many times and pathologists have widely accepted the idea that, when a culture filtrate contains a component that causes the host to wilt, this compound plays a role in pathogenesis *in vivo* (19, 21-23). The chemotherapy assay for toxin neutralization is based upon this. In brief, the method consists of growing the pathogen in liquid culture, freeing the culture medium of the pathogen, and thus obtaining a culture filtrate that contains a toxin. The toxin is then mixed with solutions of compounds under test as chemotherapeutants. If the toxin is no longer able to cause wilting under these conditions, the compound is considered potentially useful as a chemotherapeutant (15, 31).

This basic approach to chemotherapy is as valid as it ever was, but recent studies on toxic components of culture filtrates have indicated that caution must be used in applying the method. Culture filtrates are now known to contain not one but several phytotoxic components (11, 18, 20) and the role that each plays *in vivo* is obscure. Strains of a pathogen vary in their ability to produce certain of these toxins. Moreover, the yield of a given toxin, *e.g.*, polysaccharide in the case of *Ceratostomella ulmi,* can be made to vary widely by altering the medium in which the fungus is grown (15). Such discoveries have focused our attention upon the role played by phytotoxic components in culture filtrates in the infected plant. Attempts to isolate such toxins from infected plants have been attended by varying degrees of success and in these cases identity of the toxin with what is present in culture filtrates has not been shown (11, 24).

### 1. The Role of Lycomarasmin in Fusarium Wilt of Tomato

With these considerations in mind, it is of interest to consider the role played by toxins in the case of any given vascular wilt disease. Here, the discussion will be confined to the toxins of Fusarium wilt of tomato, concerning which more is known than for any other disease. Lycomarasmin has been isolated from two to four-month-old quiet cultures of *Fusarium oxysporum* f. *lycopersici*, but not from diseased plants (40, 41), and its high potency in causing pathological wilting has been the subject of a thorough study by Gaumann and co-workers (19, 21, 22, 23). It produces symptoms resembling those found in plants infected with *Fusarium* (19, 21, 49, 50). Lycomarasmin affects leafy cells by coagulating the plasma, releasing bound water. This results in increased transpiration. It also causes loss in differential permeability of the plasma membrane, with resultant loss in turgor of leaf cells (19).

There are aspects of the picture which indicate that lycomarasmin is not the sole cause of wilting in tomatoes. Thus, strains of *Fusarium oxysporum* f. *lycopersici* that are virulent may not produce as much as strains that are almost nonpathogenic (23). Moreover, tomato varieties which vary from high susceptibility (Bonny Best) to high resistance (*Lycopersicon pimpinellifolium*) are all equally sensitive to lycomarasmin (23).

Evidence that is useful in assessing the role lycomarasmin plays in infected plants has already been presented. If a compound acts chemotherapeutically by neutralizing lycomarasmin in the tomato plant, the leaf grade of treated plants should be less than would be predicted on the basis of the vascular discoloration. Among the considerable number of compounds investigated thus far (more than 300), none have lower leaf grades that would be expected in terms of the 3VO[1] grade. Such evidence is no proof that lycomarasmin is unimportant in Fusarium wilt, but at least suggests that the disease can be readily attacked through other chemotherapeutic mechanisms.

What is more pertinent in the study of the interrelations of the grades is the high degree of correlation between leaf grades and grades based on vascular discoloration. Thus, the correlation coefficient (see Section IV-B) between LG and 3VO, based on treatment means, was 0.96, while for the 105 pairs of observations (raw data), it was 0.84. Similarly, the correlation coefficient between the 105 pairs of observations of LG and 6VC was 0.95. While a high correlation coefficient does not of itself indicate a cause and effect relationship, the biology of the situation very strongly suggests either that vascular dysfunction, as measured by discoloration, causes leaf collapse or that vascular discoloration and leaf symptoms have a common cause. Gaumann (20) and Scheffer (50) have both shown that lycomarasmin does not cause vascular discoloration in tomato plants or in cuttings.

What, then, causes the discoloration? When thin freehand sections of infected tomato stems were examined microscopically, Ludwig (37) showed that mycelium of *Fusarium* could readily be detected if present.[2] By such means Waggoner (62) found vascular discoloration rather frequently in advance of tissues actually invaded by mycelium of *Fusarium*. Thus, vascular discoloration is a manifestation of the action of toxins, probably liberated by the fungus in its growth.

Both Scheffer (50) and Gaumann (20) have isolated a toxin from *F. oxysporum* f. *lycopersici* that causes vascular discoloration. It is not clear at present whether Scheffer and Gaumann are dealing with the same toxin. Scheffer's toxin from young cultures was not obtained from original culture filtrates but was produced by the recovered mycelium when resuspended in fresh nutrient for 24 hours. This filtrate caused vascular discoloration and wilting in tomatoes. The active toxin is heat labile, non-dialyzable and is precipitated by cold alcohol (50).

With these facts at hand, we may set up two hypotheses concerning the role of lycomarasmin in wilting of infected tomatoes. If vascular dysfunction, as measured in these experiments by discoloration, is proportional to wilting [3] and if lycomarasmin plays an important role in the

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[1] For a summary description of the several grading systems used in this study see Table 10, page 32.

[2] Ludwig's technique (37) consists of placing unsterilized slices of tomato stems on methylene blue-water agar and incubating them. Mycelium of *Fusarium* grows out of such sections rapidly if present in the tissue when incubated at a suitable temperature.

[3] Discoloration as such and wilting are not invariably proportional, as any field pathologist knows. Occasionally in controlled assays leaf grades are higher than would be predicted on the basis of discoloration. The question at issue is the conductive capacity of the vascular tissue. Discoloration is used only as a convenient measure of dysfunctional conductive tissue.

process but cannot cause discoloration, then lycomarasmin and the toxin responsible for vascular discoloration must be formed in a constant ratio, one to the other. Because lycomarasmin has not been isolated from the diseased plant, we must also consider another possibility: perhaps lycomarasmin is a product of *Fusarium* in culture and is not formed in infected plants.[1]

To what extent can the wilting syndrome in infected tomato plants be accounted for by vascular dysfunction alone? First, there is the evidence already presented that leaf grades and vascular discoloration are exceedingly highly correlated (Section IV-B). In addition, Ludwig (38) has studied the effect of *Fusarium oxysporum* f. *lycopersici* on the water economy of tomato plants. The transpirational pattern of diseased plants was not characteristic of what happens when the process is altered through a change in suction pressure of leaf cells. Moreover, he obtained evidence that culture filtrates are not active through their effects on water absorption by the root. Studies on water conduction through healthy vessels indicated that petiole bundles could normally conduct only 1/20 the water that stem bundles could. Detailed studies on the conductive capacities of healthy and diseased xylem soon showed that vascular dysfunction in diseased stems reduced water movement to 1/20 that in healthy stems. Ludwig observed a grey homogeneous occluding substance in unstained sections of diseased vessels. This material is evidently removed during fixation and staining of sections, since it was never seen in prepared slides (38).

The wilting mechanism in Fusarium wilt has also been studied by Scheffer[2] (49, 50). He demonstrated the impaired conductive capacity of diseased vascular elements by drawing dyes through infected and healthy stems and petioles. Dye was drawn by suction through paired segments, one healthy and one diseased, until dye had permeated the healthy segments. Vessels discolored by *Fusarium* failed to conduct the dye readily, whether in stems or petioles. If dye was forced long enough into a discolored vessel, it eventually moved through, indicating that the occluding material was not solid but was highly viscous.

Scheffer then compared the transpirational behavior of healthy and diseased tomatoes and determined that, contrary to the pattern brought on by lycomarasmin toxicity, the transpiration of infected tomato plants at first equalled that in healthy ones, but as the infection advanced and the plant became more wilted, gradually became lower and lower. In an effort to determine the extent to which a decreased transpiration might be brought on by affected leaf cells, he studied transpiration rate as a function of leaf surface and showed that as much as 1/4 of the leaf surface could be removed from a plant before any decrease in transpiration could be detected.

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[1] Scheffer (50) has calculated the disparity in lycomarasmin production in culture as compared with that needed to affect a tomato plant. Thus, he has estimated the void volume of vessels in a tomato plant one foot high as about 0.1 ml. The toxic dose of lycomarasmin for a plant this size is approximately 0.25 mg. This amount must be produced in about 10 days under optimum conditions for wilt development, although in culture *Fusarium* produces only from 1/40 to 1/100 of this amount in two months.

[2] The senior author is deeply indebted to Dr. Scheffer for his kindness in making an advance copy of his thesis (50) available during the writing of this paper.

Because lycomarasmin becomes about 10 times more toxic when it forms an iron complex (19), the disease should be more severe in plants supplied iron than it is in iron deficient plants. By growing iron deficient tomatoes and supplying them with varying amounts of this element after inoculation, Scheffer was able to detect no increase in disease severity.

Finally Scheffer isolated from young mycelium of *Fusarium* a substance that causes discoloration in vessels and makes them as resistant to movement of water and dye solutions as are vessels in diseased plants (50).

In summary, then, the work of Ludwig (38) and Scheffer (49, 50) and results reported here all indicate that an important cause of wilting in tomatoes infected with *Fusarium* is the occlusion that occurs in vessels in stems and petioles. Petioles are particularly influenced by occluding substances because their conductive capacity is low (38). Independent discoveries indicate that a toxin is formed by the fungus that is responsible for vascular discoloration (20,50,62) and occlusion of vessels as well (50). Scheffer has characterized this toxin to the extent of finding it heat labile, non-dialyzable, and precipitated by cold alcohol. Lycomarasmin, if its role in causing wilting in tomato foliage is important, is certainly not the sole cause of pathological wilting in infected plants, and in light of the evidence presented by Scheffer (49, 50) and Ludwig (38), one may wish to see it isolated from the infected plant before ascribing to it a role in Fusarium wilt of tomatoes.

### 2. The Design of Assays Based Upon Inactivation of Toxins

The foregoing discussion has been detailed because it bears upon the design of assays measuring chemotherapy through inactivation of toxins. The rigorous assay based upon this principle will deal, not with culture filtrates, but with purified toxins found to be active *in vivo*. Short of this, evaluations of compounds may subsequently be found to be artificial.

## B. DIRECT ASSAYS OF CHEMOTHERAPEUTIC POTENCY

Two types of chemotherapeutic assay may be distinguished. The indirect assay presupposes a mode of chemotherapeutic action and makes use of this assumption in accelerating the assay of compounds. The toxin assay is indirect, since it does not involve the infected plant and since, for a direct measure of chemotherapeutic activity, it is finally necessary to apply promising compounds to infected plants. The direct assay involves assessing chemotherapeutic activity upon the sick plant at once. When properly employed, the indirect assay provides information as to the mechanism by which compounds produce chemotherapeutic effects. The direct assay provides no such information.

Two direct assays have been developed for measuring chemotherapeutic potency. These are the *Botrytis* assay on *Vicia faba*, developed by Crowdy and Wain (5, 6) and the Fusarium wilt assay, described in an earlier portion of this bulletin. Because Crowdy and Wain were testing a specific hypothesis when their assay was developed, the *Botrytis*

assay has been regarded by others as an indirect assay which specifically selects systemic fungicides. This is unfortunate, since the *Botrytis* assay is in reality a direct one. Effective compounds, as measured by the *Botrytis* assay, are not necessarily fungitoxic (see Sec. VII) and may not be systemic (see below).

In briefest terms, the *Botrytis* assay consists of immersing the roots of the test plant in solutions of compounds under evaluation, or of applying compounds to soil in which plants are growing. After the plant has absorbed the chemotherapeutant, it is potted, inoculated, and after suitable incubation, the rate of development of disease is recorded on treated and check plants. In this case, the diameter of lesions on leaves is measured, and effective treatments are noted because the lesions on treated plants do not increase in diameter at the same rate as those on check plants.

Thus, the principles underlying this assay and the procedure itself are essentially the same as for the *Fusarium* assay. There are minor differences between them, however. In the *Fusarium* assay, compounds are usually absorbed by the same roots as are inoculated. In the *Botrytis* assay, absorption of chemotherapeutant takes place through the roots but leaves are inoculated. Even this difference is not inherent in the methods: chemotherapeutant may be applied as a spray in either case. Roots are generally more efficient absorbing organs than leaves. Hence, the *Botrytis* assay is better suited to measuring the ability of compounds to be translocated in the plant than the *Fusarium* assay is, and was developed in order to measure such translocative properties of compounds. However, caution must be exercised in interpreting the results as indicating that a compound has been translocated to leaves, since if the general resistance of the plant is increased, the rate of lesion development on bean leaves may be reduced. One would not interpret a comparable effect in the *Fusarium* assay as evidence of translocation.

An example of reduced susceptibility to disease resulting from localized effects is provided by the work of Keyworth and Dimond (34). Certain compounds of high efficiency in the *Fusarium* assay were observed to injure the roots of test plants. A series of compounds and physical treatments which had in common only their ability to injure roots was shown uniformly to decrease the susceptibility of the plant to wilt. Hydrogen peroxide was one such treatment, and hydrogen peroxide would be decomposed in the plant very shortly after it entered; it could by no means be conceived of as being translocated for any great distance. Root injury was accompanied by an increase in levels of reducing sugars and by a decrease in the levels of phosphate, calcium and potassium. These differences are indicative of altered metabolism in the tomato plant, an alteration which has influenced susceptibility to disease.

Treatment[1] by compounds or factors that cause root injury may influence the rate of lesion development of leaf-spotting fungi as well as the rate of invasion by vascular wilt pathogens. If so, it will be dangerous to conclude that all chemotherapeutants selected by the *Botrytis* assay are translocated to leaves.

[1] The influence of root injury upon susceptibility to toxins should likewise be considered with respect to the toxin assay for chemotherapeutants.

To the extent that the *Botrytis* assay differs from the *Fusarium* assay, each will serve as a supplement to the other in evaluating the activity of compounds and the mechanisms by which compounds reduce severity of disease in the plant. The *Fusarium* assay has been shown to be of little value in finding chemotherapeutants for Dutch elm disease. Possibly the lack of correlation in results between the *Fusarium* assay and field experiments on elms is caused by employment of a pathogen in the assay which invades the same roots as are treated. When tomato plants are inoculated at a site other than the one chemotherapeutically treated, some compounds are no longer effective against Fusarium wilt (Table 6). This suggests that the *Fusarium* assay gives preference to chemotherapeutants that themselves remain in roots or that alter the metabolism of roots without seriously affecting the plant as a whole. The *Botrytis* assay may overcome this difficulty. It is more comparable to the situation presented by Dutch elm disease in the sense that the infection court is remote from the portion of the plant to which chemotherapeutant is applied.

It is quite likely that the *Fusarium* assay may underrate the activity of compounds that are readily translocated to aboveground parts of the plant. Such compounds, if truly systemic fungicides, might accumulate in foliage and growing points but be present in only small amounts in roots. If this were true, the *Fusarium* assay would not necessarily be useful, but the *Botrytis* assay would indicate the activity of such compounds. Conversely, compounds that were substantive, remaining fixed on cellulosic material in the roots, might have little effect upon *Botrytis* on leaves but strongly inhibit the development of *Fusarium* in roots. For this reason the two assays in conjunction with one another may provide a broader basis for evaluation of chemotherapeutants than either one by itself.

## VII. Modes of Chemotherapeutic Action and the Selection of Compounds for Assay

On what basis are compounds selected for assays? This question has been asked ever since assays were first developed for chemotherapy. For the *Fusarium* assay the first compounds were selected on the basis of their high fungitoxicity, high water solubility and low phytotoxicity. These compounds had no higher frequency of chemotherapeutic potency than a random selection of compounds. Evidently there is no simple relation between chemotherapeutic potency, and chemical and biological properties of compounds. Gradually it becomes apparent that compounds are chemotherapeutants when they affect the host or the pathogen in certain specific ways and, when modes of chemotherapeutic action are known, the selection of compounds is guided by principles. To state a truism, the purpose of scientific investigation is to permit prediction; when predictions can be made, a subject has been placed upon a scien-

tific basis. Several modes of chemotherapeutic action have been recognized through experimentation and these may be used as a basis for selecting compounds for purposes of assay.

## A. ANTIDOTING THE TOXINS PRODUCED BY PATHOGENS IN INFECTED PLANTS

There seems to be little doubt that pathogens may affect the host to a significant degree through the activity of toxins. The antidoting of toxins has been discussed as an assay method. A review of current research on the role of toxins in pathogenesis indicates that such an assay method must rely upon those toxic compounds which have been shown to be produced in the infected plant. It remains for future research to disclose the nature of the toxins formed by pathogens in infected plants, and to determine the chemistry of these toxins. When the chemical nature of a toxin is known, it will be possible to select compounds for assay purposes which are thought likely to inactivate the toxin through chemical or physical reaction.

## B. INHIBITING PATHOGENS THROUGH USE OF SYSTEMIC FUNGICIDES

The work of Crowdy and Wain (5, 6) has focused our attention upon systemic fungicides as chemotherapeutants. Their approach was influenced by the work of Schrader, who worked in Germany on organic phosphates as nerve poisons, and developed compounds now known as parathion, Systox and Pestox. The latter two compounds were taken by Allied research teams back to Great Britain for research in the agricultural field. It was subsequently shown by Ripper, Greenslade and Hartley (45) that when these compounds are sprayed on foliage or absorbed by roots of plants, they are insecticidal to sucking insects. This work aroused an interest in systemic insecticides and it was natural to seek compounds that were systemic fungicides.

Wain had explored the relation of structure to activity among compounds having hormone activity in plants and had noted the structural modifications that rendered such compounds nonphytotoxic (and inert as hormones). Presumably hormones with a slight structural modification were still translocatable in plants. A series of compounds lacking hormone activity was investigated by Crowdy and Wain with respect to their fungitoxicity. Those that were both fungitoxic and translocatable were applied to roots of *Vicia faba,* which was subsequently inoculated with *Botrytis* in an effort to determine whether or not the compounds acted as systemic fungicides. Those that were fungitoxic reduced the rate at which lesions caused by *Botrytis* developed on leaves (5, 6).

The work of Crowdy and Wain has provided us with a rational basis for selecting compounds for assays, while contributing a distinctive concept to chemotherapy. Many compounds that occur naturally in plants are unlikely to be destroyed rapidly by host metabolism. When slight modifications are made in such compounds to render them fungitoxic, they may be useful in combating plant pathogens. Guided by these

principles, one is provided with a rational basis for chemotherapy of one type.

Antimetabolites have served as a basis for research in animal chemotherapy, but have been little used in control of plant diseases. Many fungi are known to be deficient in their ability to produce growth factors. For example, *Ceratostomella ulmi* lacks the ability to produce pyridoxine. By subjecting the host to treatment with an antipyridoxine factor, it ought to be possible to reduce the severity of Dutch elm disease, if the host is neither exceptionally capable of supplying this factor to its pathogen nor drastically affected by treatment with an antipyridoxine factor. As yet, such growth factors cannot be produced in sufficient quantity economically to be used as chemotherapeutants for plant diseases. Similarly, fungi autotrophic in growth-factor production may be inhibited by the use of antimetabolites in the host. Obviously any antimetabolite that is useful in chemotherapy must be a systemic fungicide, but the criteria for selecting compounds for assays will differ somewhat from those used for selecting systemic fungicides in general.

## C. RELATING FUNGITOXICITY TO CHEMOTHERAPEUTIC POTENCY

If fungitoxic mechanisms are frequently involved in chemotherapy, there ought to be a correlation, however slight, between fungitoxicity of compounds and their chemotherapeutic potency. Studies on the chemotherapy of crown gall, in which a series of dyes was used, indicated that there was no clear relation between toxicity of compounds to *Aerobacterium tumefaciens* and the chemotherapeutic potency of dyes (13).

In an effort to examine this relationship more critically, a study was made of the fungitoxicity of more than 100 compounds whose chemotherapeutic potency had been assayed. By using the glass slide technique developed by Horsfall (26), the ED 50 values (concentrations necessary to inactivate 50 per cent of the spores) of compounds were obtained for two fungi, *Stemphylium sarcinaeforme* and *Sclerotinia fructicola*. When these values were plotted in logarithms against the chemotherapeutic potency, expressed as the ratio of the mean disease levels in treated plots to the mean disease levels in check plots, scatter diagrams were obtained which are shown in Figure 12 for *Stemphylium* and Figure 13 for *Sclerotinia*.

It is readily apparent from these figures that there is no relationship whatsoever in a population of compounds between the toxicity of compounds to either fungus and their chemotherapeutic potency. Many compounds that are highly fungitoxic have no chemotherapeutic activity and chemotherapeutic compounds are not necessarily fungitoxic. Clearly, nothing is gained in chemotherapeutic assays by a preliminary assay of fungitoxicity. If it is necessary to reduce the number of compounds to be assayed, one might as well arbitrarily eliminate a stated fraction of the compounds at the outset as to select only those compounds which are fungitoxic.

However, one may obtain useful information from a survey such as that recorded in Figures 12 and 13. Those compounds that are highly

fungitoxic and also have high chemotherapeutic potency may be active as systemic fungicides. Such a correlation may therefore narrow the search for compounds that are active as systemic fungicides.

The relation of fungitoxicity to chemotherapeutic potency has been studied further by Davis (7). He measured the toxicity of five unrelated compounds of varying chemotherapeutic potency to mycelium of *Fusarium oxysporum* f. *lycopersici* when they were added to liquid medium.

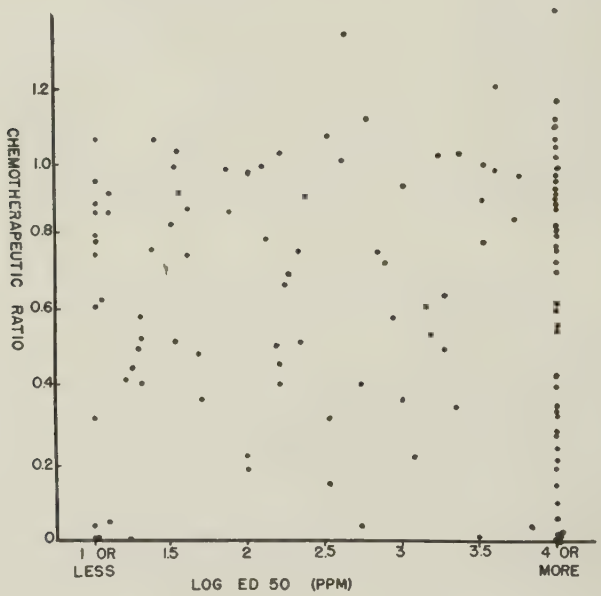


**Figure 12. A scatter diagram indicating the relation between fungitoxicity of compounds to Stemphylium sarcinaeforme and their chemotherapeutic potency.**

A further estimate of toxicity was made by measuring the effect of these compounds on oxygen uptake by mycelium of *Fusarium* in Warburg vessels. Two compounds, HD 109, the potassium salt of 2-carboxymethyl mercaptobenzothiazole and HD 139, 2-(*n*-amyl)-pyridine, were effective as chemotherapeutants at concentrations from 1/40 to 1/10 of those required to inhibit growth of mycelium or oxygen uptake by respiring cells. Two others, CC 1182, 4-chloro-3,5-dimethyl phenoxy ethanol, and HD 3, octadecyl trimethyl ammonium pentachlorophenate, were inhibitory to both respiration and growth of mycelium at concentrations which were effective as chemotherapeutants. 8-Quinolinol benzoate, a mediocre chemotherapeutant for tomato wilt, was inhibitory to growth of mycelium at concentrations 1/20 of that required to produce a moderate reduction in disease severity in tomatoes when used as a chemotherapeutant. On this basis Davis has concluded that there is no essential relation of fungitoxicity to chemotherapeutic potency (7).

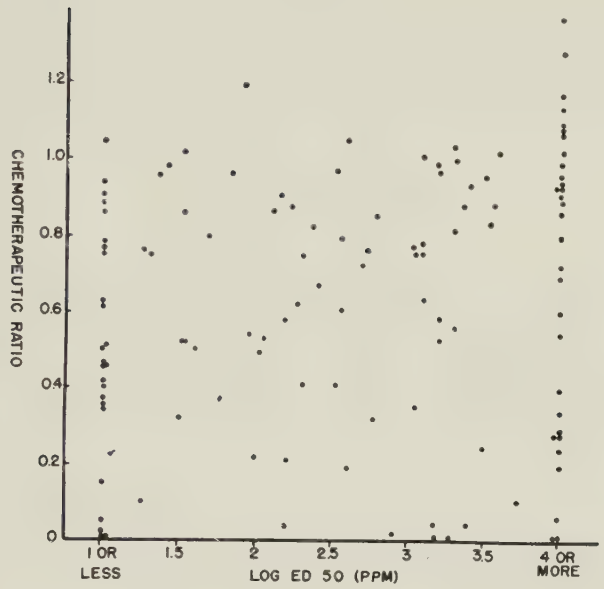


**Figure 13. A scatter diagram indicating the relation between fungitoxicity of compounds to Sclerotinia fructicola and their chemotherapeutic potency.**

Similar information was obtained by Dimond and Davis (10) for the sodium salt of 2-carboxymethyl mercaptobenzothiazole. The ED 50 value of this compound against spores of *Stemphylium* and *Sclerotinia* in slide germination experiments was found to be of the order of 10,000 ppm. However, a dosage-response curve for chemotherapeutic activity (Curve B in Figure 4) indicated that the concentration for 50 per cent reduction in disease severity was of the order of 10 ppm. Obviously, even if the compound were accumulated by the plant at levels above the concentration at which it is applied, it would be unlikely to attain concentrations in the plant that were toxic to fungi. Because the estimates of fungitoxicity were based on fungi other than the pathogen involved, toxicity to *Fusarium* was also determined. In this case the compound was added to liquid nutrient in which the fungus was grown. The concentrations necessary to inhibit *Fusarium* were from 20 to 50 times greater than the maximum concentration that just failed to be phytotoxic to tomatoes. Finally, the sap of treated plants was tested for its fungitoxicity and none was found. Apparently the compound is not active by virtue of its fungitoxicity.

Such an approach may be helpful in determining whether or not other compounds act as systemic fungicides. If compounds do not accumulate in leaves at fungitoxic levels and do not occur in fungitoxic concentrations in root or stem tissues, they are unlikely to be active as fungicides in the plant.

There are reasons why one would not expect a high correlation of fungitoxicity with chemotherapeutic potency. Considered in terms of the *Fusarium* assay, some fungitoxic compounds may fail to enter the plant and others may be broken down rapidly in the plant to non-fungitoxic products. In terms of the *Botrytis* assay, substantive compounds may be inactive while only those which are readily translocated will be active.

## D. ALTERING RESISTANCE OF THE HOST TO THE PATHOGEN

### 1. General Evidence

It has long been recognized that the plant may be varied in its resistance to disease through altering its nutrition. In Fusarium wilt of tomatoes, susceptibility is markedly altered by varying the balance and concentration of nutrients (58, 64). Verticillium wilt on hops (35) and on tomatoes (47) may be reduced in severity by withholding nitrogen from the plant. Such work suggests immediately that these relations may be useful in plant chemotherapy.

Reducing sugars levels in plants likewise are correlated with susceptibility to disease in a number of cases. Such effects have been observed by Rowell (48) on *Alternaria* blight of tomato, by Feldman, Caroselli and Howard (15) on Dutch elm disease and by Eaton and Rigler (14) for cotton root rot.

2,4-Dichlorophenoxy acetic acid drastically affects the levels of reducing sugars in plants (65) and it was recently learned that this compound may significantly alter the nitrate levels in leaves of certain plants (54). Therefore, applications of 2,4-dichlorophenoxy acetic acid may be expected to have an effect upon the resistance of the plant to disease. Recent reports of the effect of 2,4-D upon susceptibility of plants to disease (32, 33) support such a contention. Rowell (48), in fact, employed this compound deliberately to make plants susceptible[1] to disease.

Host metabolism may surely be altered in other ways to produce plants that are resistant to disease (51). Changing nitrate and reducing sugar levels in the host are but two of many possible ways of changing susceptibility to disease. Undoubtedly it will be convenient to regulate the metabolic balance in the plant through applying compounds that influence metabolism. Compounds which, through such action, produce a more resistant host, are chemotherapeutants.

### 2. Root Injuring Compounds As Chemotherapeutants

An example of how compounds may alter host metabolism, and thus, susceptibility to disease, is provided by the work of Keyworth and Dimond (34). Certain chemotherapeutants were observed to injure the

[1] Since pathogens differ in their attack upon the host, an increase in reducing sugar levels in the host may suppress disease or make it worse, depending upon the disease involved. Inverse chemotherapeutic effects have frequently been noted in the Fusarium wilt assay. The most startling case has been described by Waggoner and Dimond (63) who used maleic hydrazide to stunt tomatoes to the same extent as Fusarium wilt stunts them. Plants that were treated with maleic hydrazide and inoculated suffered far worse from Fusarium wilt than inoculated controls did.

roots of plants to which they were applied. By treating plants with a series of compounds such as NaCl and $H_2O_2$, which had little in common other than their ability to injure roots, they showed that whenever the roots are injured, the plant becomes more resistant to infection by *Fusarium*. Reducing sugar levels are markedly increased, while certain nutrient elements were present in smaller amounts in plants with injured roots. It was suggested that the effect of root injury is to alter metabolism of the host in such fashion that it becomes more resistant to disease (34).

Probably the compounds that injure roots seriously will fail as chemotherapeutants in practice when used at injurious concentrations. It is likely, however, that other compounds will alter metabolism in similar fashion without serious injury to the plant. These compounds may be practical as chemotherapeutants. This possibility is suggested by work of Davis and Dimond (9) in which nonfungitoxic compounds that alter reducing sugar levels were applied to leaves of plants, and after absorption into the plant acted as chemotherapeutants for Fusarium wilt of tomato.

### 3. Altering Host Metabolism and Resistance Without Injury

Davis (7) and Davis and Dimond (8) have studied the probable mechanisms by which two highly effective chemotherapeutants act. These compounds do not cause root injury. The methods used in studying the relation of fungitoxicity to chemotherapeutic potency have already been discussed. Tomato plants were treated with CC 1182, 4-chloro-3,5-dimethyl phenoxy ethanol, and HD 160, the sodium salt of 2-carboxymethyl mercaptobenzothiazole. Plants were harvested and analyzed for (a) reducing sugar content, (b) a number of water soluble nitrogen fractions and (c) the rate of oxygen uptake of tissue macerates, all relative to those in check plants. CC 1182 treated plants were found to differ appreciably from checks on all counts. HD 160 treated plants differed only in water soluble nitrogen fractions. Thus, a marked change in host metabolism has been brought on as a result of treatment, a change which resulted in plants that were more resistant to Fusarium wilt. Evidence that these compounds are not present in fungitoxic concentrations in the plant has already been presented.

It is as yet uncertain which mechanisms in the host can be changed to reduce their susceptibility to disease. The evidence suggests that reducing sugar levels are involved in susceptibility to disease and that by changing them specifically, it may be possible to produce plants with altered resistance to Fusarium wilt. If such changes can be produced with compounds applied to roots or foliage, these compounds will be chemotherapeutants.[1]

[1] On page 20 data were presented which suggest that CC 1182 is no longer effective as a chemotherapeutant when plants are inoculated aboveground and roots are treated. On the other hand, this compound influences reducing sugar levels of treated plants (7). Evidently CC 1182 alters metabolism of the host and when applied to roots the effect on resistance is greater in roots than in aerial portions of the plant.

### 4. The Selection for Assay Purposes of Compounds That Alter Host Metabolism

It appears at present as though compounds that alter reducing sugar levels will also alter resistance of plants to disease. Perhaps no group of compounds so strongly influences reducing sugar levels in plants as those that act as hormones. It will be worthwhile to bear this in mind in selecting compounds for assay purposes. Our own studies have shown a high degree of correlation between compounds with formative effects in plants and their chemotherapeutic potency. Thus, the compound 4-chloro-3,5-dimethyl phenoxy ethanol and the K and Na salts of 2-carboxymethyl mercaptobenzothiazole are as effective chemotherapeutants in the *Fusarium* assay as any compounds yet encountered and each will produce formative effects upon tomatoes at sufficiently high concentrations. Davis and Dimond (9) have explored the chemotherapeutic activity of a number of compounds as varied as 2,4-dichlorophenoxy acetic acid, indole acetic acid, naphthalene acetic acid, $\beta$ naphthoxy acetic acid, 2,3,5-iodobenzoic acid, and chloro-*o*-isopropyl phenyl carbamate. All of these increased the resistance of the plant to Fusarium wilt and correspondingly decreased the reducing sugar levels. This was adequately demonstrated in an experiment in which growth hormones were applied to foliage as chemotherapeutants and reduced disease levels in the plant as a whole. The balance between increasing resistance and increasing susceptibility to disease through altered metabolism may be delicate. Thus, maleic hydrazide has been found by Waggoner and Dimond (63) to cause the plant to become strikingly more susceptible to Fusarium wilt, and it, too, exerts a profound effect upon the metabolism of the plant to which it is applied.

## E. SELECTING COMPOUNDS AT RANDOM FOR ASSAY PURPOSES

There are probably a number of ways in which compounds may act chemotherapeutically which are not yet understood. These mechanisms may be recognized deductively in the future or they may be arrived at empirically. There is every reason to continue an empirical search for compounds having chemotherapeutic activity. Thus, we cannot say at the outset whether or not a preconceived mode of chemotherapeutic action is likely to succeed in practice and it will be useful to determine in direct assays which kinds of chemotherapeutic activity succeed best. Having assembled a number of useful compounds, it will then be profitable to explore the mechanisms by which they produce their effects. In such manner will be developed an appreciation of which chemotherapeutic mechanisms are most frequently effective and also which ones produce the greatest reduction in disease severity. With this approach, for example, the reduction of disease by chemotherapy through affecting host metabolism has been developed.

## VIII. Summary and Conclusions

1. Chemotherapy is defined as the control of plant disease by compounds which, through their effect upon the host or pathogen, reduce or nullify the effect of the causal agent after it has entered the plant (28). The compound which is applied to the plant for this purpose is called a chemotherapeutant.

2. An assay method has been developed for measuring chemotherapeutic potency of compounds. This method consists of applying compounds in solution or suspension at the maximum concentration that causes no visible injury to the plant. Application is made each day for a standard treatment period. Bonny Best tomatoes are treated by applying the solution to the roots, which then absorb the compound if possible. Plants are uprooted after treatment, the roots washed, wounded, and inoculated with *Fusarium oxysporum* f. *lycopersici* by immersing the roots in a dense suspension of bud cells, produced in shake culture. Plants are then repotted in fresh sand and in a pot that is free of chemotherapeutants. After a suitable incubation period, plants are graded for severity of disease by counting the number of large vascular bundles in the stem that are discolored by *Fusarium*. A disease index is computed for the plant by measuring discoloration at each internode up the stem of the plant and dividing the number of discolored bundles by the total number of bundles observed. In order to compare compounds from one experiment to another, the chemotherapeutic ratio must be computed. This is the ratio of the disease index in treated plots to that in check plots.

3. The experimental basis for the assay method is presented. Plants must generally receive treatments before inoculation. Treatment effects are more pronounced and more uniform when they are applied to the root system than when the compounds are injected into the stem with a hypodermic needle. Beyond a definite treatment period before inoculation, there is little increase in chemotherapeutic effect. Since bud cells produced in shake culture in four days contain negligible quantities of phytotoxic compounds, they need not be washed before being used as inoculum. Plants inoculated by dipping injured roots into the inoculum become more uniformly diseased than they do when they are inoculated at the ground line by means of a hypodermic needle.

4. Varieties of tomato that are moderately resistant to Fusarium wilt rank compounds in the same order as varieties that are highly susceptible.

5. Concentration series of effective compounds produce a graded response in severity of disease. The logarithm of concentration of chemotherapeutant applied is directly proportional to probit of the disease index. Therefore, one is justified in applying compounds in the assay at the maximum concentration that does not reduce vigor of the plant below that in checks in preliminary evaluation. When chemotherapeutic potency does not decrease with the concentration at which

compounds are applied, the compound may have been employed at levels that were so phytotoxic as to cause errors in estimating the chemotherapeutic potency.

6. Some chemotherapeutants are inactivated in soil while others are not. For this reason, although compounds are first assayed on plants grown in sand, they should be reevaluated in plants grown in soil before being used in field experiments.

7. Chemotherapeutic effects are generally short lived. Treatments must be repeated from time to time to maintain resistance of the plant.

8. Compounds are shown to be absorbed by the plant and the reactions that result in a resistant plant occur within the host. These are the conditions that are defined as chemotherapeutic.

9. Eight methods of grading severity of Fusarium wilt are appraised. For discriminating treatment differences, the 3VO grading method, which is used in the standard assay method, is superior in a number of respects to any of the other grading methods. It is based upon counting the number of large vascular bundles that are discolored in each internode, summing them for the entire plant and dividing by the total number of bundles observed. The next most useful method of grading disease severity consists of grading leaves for severity of wilt on a scale from 0 (unaffected) to 4 (leaf completely nonfunctional or missing). The leaf grade is more subject to positional and seasonal effects than is the 3VO grade and a number of factors may affect the response of leaves other than disease itself.

10. Interrelations of these grading methods have been studied. There is very high correlation indeed between the severity of vascular discoloration in petioles or stems and the severity of wilting symptoms in leaves. It is concluded that all grading methods are measures of one another, some more accurately than others. Therefore, nothing can be learned concerning the mode of action of chemotherapeutants by a study of the interrelations of these several grading systems, so far as present evidence indicates.

11. Chemotherapy assays are useful in relating chemical structure to chemotherapeutic activity. For a series of benzene sulfonanilides, the relation of structure to activity is illustrated.

12. Assays are also useful in selecting compounds with high chemotherapeutic activity which are unrelated chemically. The mode of chemotherapeutic action of each can then be studied with a view to learning (a) what modes of action are most frequent among compounds and (b) what modes of action give greatest chances of practical use.

13. Assays are valuable for development of chemotherapeutants in practice, only if the performance of compounds can be predicted in field

use from their behavior in assays. Soil borne vascular wilt diseases such as Fusarium wilt of carnations and Verticillium wilt of eggplant can be controlled by the same compounds as are found effective in the tomato assay. There is a significant correlation between the order of effectiveness of compounds as measured by the tomato assay and the order of their performance in the field on these diseases. As yet, the results of the tomato assay are not useful in predicting the performance of compounds when used as chemotherapeutants for Dutch elm disease.

14. Two types of chemotherapeutic assay are distinguished: the direct assay which measures the ability of compounds to act chemotherapeutically on the infected plant and the indirect assay which presupposes a mode of chemotherapeutic action and permits accelerated evaluation of compounds through use of this assumption. Toxin assays (15) are of the indirect type, whereas the *Fusarium* assay and the *Botrytis* assay, (5, 6) are direct.

15. Toxin assays must deal with purified toxins whose role in the infected plant has been demonstrated. As an example, the role of lycomarasmin in Fusarium wilt of tomato is discussed; it is concluded that basing an assay upon inactivation of lycomarasmin must await the demonstration of lycomarasmin in infected plants.

16. Two direct assays are compared with one another. It is concluded that the *Botrytis* assay (5, 6) may measure properties of compounds which the *Fusarium* assay does not and *vice versa.* This suggests that the two assays may supplement each other well in chemotherapy research.

17. The mechanisms by which chemotherapeutants produce their effects serve as a rational basis for choosing compounds for purposes of assay.

18. When toxins are to be inactivated in assays and their structure is known, compounds may be selected on the basis of their presumed reaction with the toxin to inactivate it.

19. Systemic fungicides have already been selected by Crowdy and Wain (5, 6) on the basis of their translocatability in plants and their fungitoxicity. Antimetabolites are types of systemic fungicides and may be selected, using slightly different criteria.

20. There is no over-all relation between fungitoxicity of compounds and their chemotherapeutic potency. However, those compounds which are both highly fungitoxic and efficient as chemotherapeutants may act as systemic fungicides.

21. The resistance of plants to disease may be altered through their nutrition and through altering their levels of reducing sugars. In gen-

eral, compounds that cause root injury increase the resistance of tomatoes to Fusarium wilt (34).

22. Host metabolism may be altered without serious injury by employing compounds which change the metabolic pathways that govern disease resistance. Growth hormones generally affect reducing sugars levels in plants and they also generally affect resistance to disease (9).

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